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SURVIVAL OF *Klebsiella pneumoniae (sensu lato)* AT LOW TEMPERATURES

by

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DEDICATION

Dedicated to

God, and my mother, Kathleen.

Without them, this thesis would not have been.

ABSTRACT

The effect of refrigeration and freezing on survival of *Escherichia coli* and *Klebsiella pneumoniae* (*sensu lato*) stored in different suspending media (broths) at 10, 4 and -16°C and in ground beef at 4, -16 and -40°C, was studied. In the broth media, survival of the test cultures was greater at 10 and 4°C than at -16°C. Death of up to 4 log cycles (99.99 percent) of the cells occurred in 10% Tryptic Soy Broth and Nutrient Broth at -16°C. In contrast, survival of the test cultures in ground beef was greater at -16 and -40°C than at 4°C. Several factors which may have contributed to the observed decreases in the population were examined. These included changes in biochemical characteristics, the influence of different pH levels and the influence of a competitive meat microflora.

Tests based on the ability of coliforms and faecal coliforms to ferment lactose with production of gas, and to produce indole from tryptophan at 35 and 44.5°C were performed to determine whether changes in biochemical characteristics occurred as a result of low temperature storage. No permanent variants were obtained that were negative for these characteristics. However, some permanent variants were obtained that were negative for the production of gas from lactose fermentation or the production of indole from tryptophan at different maximum temperatures. There were no marked effects on the survival of the test cultures that could be attributed to pH, a competitive aerobic or an

anaerobic microflora.

From these observations, it appeared that *E. coli* and *K. pneumoniae* (*sensu lato*) died as a result of storage temperature and time, and not as a result of pH or a competitive meat microflora. These observations have important implications for the food industry and the use of either *E. coli* or *K. pneumoniae* (*sensu lato*) as indicators of unsanitary conditions in meats.

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List of Abbreviations

CFU	Colony Forming Units
MCIC	MacConkey Inositol Carbenicillin Agar
NB	Nutrient Broth
TBA	Tryptone Bile Agar
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSI	Triple Sugar Iron Agar
VRBA	Violet Red Bile Agar
TSA/VRBA	Tryptic Soy Agar overlayered with VRBA

I. INTRODUCTION

The aim of the food industry is to provide the consumer with a supply of food that is of sound sanitary quality; that is, free from pathogenic microorganisms.

Most food poisoning outbreaks in Canada, the United States and Europe are caused by *Staphylococcus aureus*, *Salmonella* spp., and *Clostridium perfringens*. (Buttiaux & Mossel, 1961; Miskimin *et al.*, 1976; Todd, 1978). There are difficulties associated with the routine examination of food for pathogenic microorganisms. Pathogens may occur in low numbers in contaminated food and may be unevenly distributed, thereby making them difficult to isolate. Other harmful organisms, including viruses and intestinal parasites can be present in foods. Reliable methods for their detection may not be available to the food microbiologist, or they may be too complex for routine examination (Buttiaux & Mossel, 1961; ICMSF, 1978).

It was necessary, therefore, to find methods which would detect and help control the spread of food-borne pathogens. Microbiological methods which were proven to be of value in non-food systems, such as water, were adapted for use in foods, sometimes with questionable success (Miskimin *et al.*, 1976). These methods included the use of "indicator" organisms. The basis for using indicator organisms was to reveal unsanitary practices, for example, temperature abuse, which contribute to deterioration in food quality, and to reveal conditions indicative of a potential

health hazard (ICMSF, 1978). The indicator organisms most commonly used are the coliform bacteria (especially the faecal coliforms), *E. coli*, *C. perfringens* and Lancefield group D streptococci which are loosely referred to as "faecal streptococci" (Buttiaux & Mossel, 1961; ICMSF, 1978; Kott, 1977). Other indicator organisms occasionally used include *S. aureus*, plaque-forming enteroviruses and coliphage (ICMSF, 1978; Pipes, 1982).

Research dealing with indicator organisms and their use in the detection of contamination of foods focuses mainly on contamination by enteric pathogens (that is, faecal contamination), the most important of which are *Salmonella* and *Shigella*. The idea is that if indicator organisms of the same family and similar habitat and characteristics are absent from foods, then it is reasonable to assume that pathogenic organisms are also absent (Buttiaux & Mossel, 1961; ICMSF, 1978). An indicator organism of faecal contamination may best be defined (Kott, 1977) as:

> ....a biotype that is prevalent in sewage and excreted by humans or warm-blooded animals. In addition, the indicator should be present in greater abundance than pathogenic bacteria, incapable of proliferation - or at least not more capable than enteric bacteria, more resistant to various disinfectants than the pathogenic bacteria, and quantifiable by simple and rapid laboratory procedures.

Of the known indicator organisms, the coliform bacteria and *E. coli* are considered to be most suitable and are the most commonly accepted indicators (Kott, 1977). The inadequacies of the other indicator organisms are seen in

their low recovery rates and poor enumeration methods (Evans, 1977; ICMSF, 1978; Niven, 1963).

The coliform bacteria are

> .....aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria which ferment lactose with gas formation within 48 hr at 35 C (Greenberg *et al.*, 1981).

They include members of the family Enterobacteriaceae such as species of *Escherichia*, *Klebsiella*, *Enterobacter* and *Citrobacter*. Of these four bacteria, only *E. coli* is considered to be an exclusive inhabitant of the intestinal tract. *Klebsiella*, *Enterobacter* and *Citrobacter* also occur in the intestines, but in much lower numbers. They are also widely distributed elsewhere in the environment; for example, on vegetation and in soils (Buttiaux & Mossel, 1961). In studies on the distribution of these "coliform" bacteria in human faeces (Dufour, 1977), it was found that *E. coli* represented 96.8 percent of isolates, *Klebsiella* spp., 1.5 percent and the *Enterobacter-Citrobacter* group 1.7 percent of the isolates.

The coliform bacteria have been divided into faecal and non-faecal types. The coliforms of probable faecal origin are those that ferment lactose with gas production at 44-45.5±0.2°C within 48 hours (ICMSF, 1978). Two genera fall into this category, *Escherichia* and *Klebsiella*. The fermentation of lactose with gas production at elevated temperatures is the only known characteristic used to distinguish between faecal and non-faecal coliforms.

However, it is an arbitrary definition, since some members of the coliform group may be isolated from non-faecal sources and still possess the ability to ferment lactose with gas production at 44-45.5°C. Most emphasis has been placed on faecal coliform bacteria as indicators of unsanitary conditions; however, non-faecal coliforms may also be useful as indicator organisms. Non-faecal coliforms make up about a third of the coliform bacteria present in raw sewage (Dufour, 1977), therefore, they may indicate contamination by raw sewage or untreated water, so that their presence in food and water may also be of sanitary significance.

E. coli does not proliferate in water, in fact it dies. Therefore, the presence of even one faecal coliform in 100 ml of untreated water is considered to be significant by public health authorities. Treated water should not contain coliform bacteria. The sensitivity of the coliform bacteria to bactericidal agents is similar to that of the potential pathogens, *Salmonella* and *Shigella* (Buttiaux & Mossel, 1961). The same significance cannot be applied to coliform bacteria in foods. In fact, coliform bacteria may form part of the natural flora of foods such as vegetables and raw meats (Buttiaux & Mossel, 1961; Geldreich *et al.*, 1964; Remington & Schimpff, 1981; Wright *et al.*, 1976). Patterson and Woodburn (1980) reported total coliform bacterial counts on alfalfa and bean sprouts of 3.2×10^6 and 1.9×10^6 cfu/g, respectively. The majority of the isolates were

Klebsiella pneumoniae (*sensu lato*). Other researchers have also reported the association of coliform bacteria with the botanical environment (Brown & Seidler, 1973; Cooke *et al.*, 1980; Knittel *et al.*, 1977).

Raw meats contain *E. coli* and other coliform bacteria, even with good processing practices. A major source of meat contamination by coliform bacteria is usually the hide of animals during processing (Newton *et al.*, 1977). Meat supports the growth of *E. coli* and other coliform bacteria when stored improperly. A high coliform count in raw meat, therefore, may not be a reliable indicator of sanitary quality. Furthermore, the ability of coliform bacteria to grow in meats contravenes Kott's definition of an indicator organism.

The use of *Klebsiella* spp., in particular *K. pneumoniae*, as indicators of unsanitary conditions was suggested by Bagley and Seidler (1977). However, the use of *K. pneumoniae* as a faecal indicator has been challenged, since, unlike *E. coli*, it is ubiquitous and not restricted to the intestinal tract. Nevertheless the following points about *K. pneumoniae* should be considered. Sixteen percent of environmental isolates and 85 percent of known pathogenic *K. pneumoniae* had biochemical characteristics indicating that they were faecal coliforms (Bagley and Seidler, 1977). This suggests that isolates from both clinical and environmental sources may have once been from a common (probably faecal) origin. Several authors have reported that environmental

isolates are phenotypically and serologically indistinguishable from isolates associated with human and animal infections (Rennie and Duncan, 1974). The sanitary significance of these organisms, therefore, regardless of their origin, should not be neglected.

The occurrence of Enterobacteriaceae in raw meats and their significance at different stages of the meat handling process was studied by Stiles and Ng (1981). They observed that *E. coli* biotype I, *K. pneumoniae* and *Serratia liquefaciens* were the dominant Enterobacteriaceae isolated from the raw meat handling areas of packing plants. Analysis of raw meats at retail level, before handling by butchers, showed that the dominant Enterobacteriaceae had changed to *S. liquefaciens*, *Enterobacter agglomerans* and *E. coli* I. *K. pneumoniae* was identified as a minor isolate. The conclusions that were drawn from these observations were: (i) the frequent occurrence of *E. coli* biotype I in raw meat such as ground beef at both packing plant and retail levels limits the value of this organism as an indicator of sanitary conditions of meat; (ii) infrequent isolation of *K. pneumoniae* at retail level suggests the possibility that this organism has a short survival time in meats, so that its presence at retail level could indicate recent contamination.

II. OBJECTIVES

(i) To determine the survival of *K. pneumoniae* and *E. coli* in (a) liquid laboratory media and (b) ground beef at low storage temperatures.

(ii) To determine whether phenotypic changes occur as a result of low temperature storage.

(iii) To determine whether pH and indigenous meat microflora influence survival of *K. pneumoniae* and *E. coli* at low temperatures.

III. REVIEW OF THE LITERATURE

A. The Genus *Klebsiella*

Taxonomy

The present definition of the genus *Klebsiella* according to the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1975) is as follows:

> Non-motile, capsulated rods, 0.3-1.5 um by 0.6-6.0 um, arranged singly, in pairs or short chains. Grow on meat extract media producing more-or-less dome-shaped, glistening colonies of varying degrees of stickiness, depending on the strain and the composition of the medium.
>
> No special growth requirements and most strains can use citrate and glucose as sole C source, and ammonia as N source. Glucose is fermented with the production of acid and gas (more CO_2 than H_2), but anaerogenic strains occur. Most strains produce 2,3-butanediol as a major end product of the fermentation of glucose and the V.P. reaction is usually positive; lactic, acetic and formic acids are formed in smaller amounts and ethanol in larger amounts than in a mixed acid fermentation. H_2S is not produced from TSI; gelatinase and indole are usually not produced.
>
> Optimal temperature for growth 35-37 C; optimal pH about 7.2.

This definition is the result of many years of research on this very adaptable organism. At present, Bergey's Manual (Buchanan & Gibbons, 1975) recognises three species: *K. pneumoniae*, *K. rhinoscleromatis* and *K. ozaenae*. The latter two species have definite characteristics which allow them to be readily identified. *K. pneumoniae* has less definite characteristics which has made its classification more

difficult. It was first isolated by Friedländer in 1883 (Hay, 1932). It was recovered from patients suffering from pneumonia and described as a capsulated bacillus. This led the way to the discovery of other capsulated bacilli from pathological lesions and a wide range of other environments. However, these organisms were not identical in every respect with Friedländer's bacillus, and consequently, they were given different names (Perkins, 1904). As a result a large variety of names was used for virtually the same organism. In 1896, Fricke gathered all of these organisms into one group that was called *Bacillus mucosus capsulatus* (Hay, 1932).

One organism which researchers were reluctant to include in this group was *Bacillus lactis aerogenes*, because when it was first isolated by Escherich in 1886, he emphasized its similarity to *B. coli* (now known as *Escherichia coli*), and as a result, its similarities to Friedländer's bacillus went unnoticed (Hay, 1932).

Perkins (1904) classified the *B. mucosus capsulatus* group according to certain biochemical reactions, as follows:

1. *Bacterium aerogenes* - fermented dextrose, lactose and saccharose with gas formation.

2. *Bacterium pneumonicum* (Friedländer's bacillus) - fermented dextrose and saccharose but not lactose with gas formation.

3. *Bacterium acidi lactici* - fermented dextrose and lactose but not saccharose with gas formation.

Perkins (1904) also noted that the third group, *B. acidi lactici*, was generally called *B. aerogenes*. Later *B. aerogenes* was described as the type culture for *B. mucosus capsulatus*; the other two organisms were variants which had lost the ability to ferment certain carbohydrates (Perkins, 1907).

For many years, *B. aerogenes* and Friedländer's bacillus were classified separately. During this time, *B. aerogenes* became known variously as *Aerobacter aerogenes*, *B. lactis aerogenes*, *B. pneumoniae* (Buchanan & Gibbons, 1975). Over the years, researchers endeavoured to define the relationship between *B. aerogenes* and Friedländer's bacillus. One of the first studies was done by Edwards (1929). The characteristics he used to compare the two organisms included biochemical, fermentative and serological reactions. Based on his observations, Edwards (1929) concluded that:

> The organisms .... received from various sources labelled Friedländer bacilli cannot be distinguished from *Bact. aerogenes* and other members of the encapsulated group.... *Bact. aerogenes* is so closely related to the other encapsulated forms that they should be classified in the same genus. No constant differences have been observed which could be used to separate the organisms into two or more species.

Later, in support of Edwards (1929), non-motile strains of *A. aerogenes* and Friedländer's bacillus were combined into the *Klebsiella* group, while motile *Aerobacter* spp. were

renamed *Enterobacter aerogenes* and *E. cloacae* (Edwards and Ewing, 1962).

Research favouring the separate classification of *A. aerogenes* and Friedländer's bacillus goes as far back as the early 1930s. Hay (1932) reported that, within the *B. mucosus capsulatus* group, there were serological differences between faecal and respiratory tract isolates. As a result, she described two types: faecal (*B. lactis aerogenes*) and non-faecal (Friedländer's bacillus).

In support of Hay (1932), Duguid (1959) reported that *A. aerogenes* was fimbriate while *Klebsiella* spp. were not. Based on fimbriation and certain biochemical reactions, Cowan *et al.* (1960) proposed the following division of the genus *Klebsiella*: *K. pneumoniae*, the type species; *K. aerogenes*, formerly *A. aerogenes*; *K. rhinoscleromatis*; *K. ozaenae*; *K. edwardsii* var. *edwardsii* and var *atlantae*. This classification was confirmed by Ślopek and Durlakowa (1967), with only slight variations. They concluded that the two varieties, *K. edwardsii* var *edwardsii* and var *atlantae* were similar to *K. aerogenes* and *K. pneumoniae*, respectively and differed only with respect to fimbriation. Therefore, they proposed that the varieties *K. edwardsii* and *K. atlantae* be subspecies of *K. aerogenes* and *K. pneumoniae*, respectively and renamed them *K. aerogenes* var *edwardsii* and *K. pneumoniae* var *atlantae*.

Within the *Klebsiella* group, there are a number of cultures that liquefy gelatin and produce indole. *K. oxytoca*

was first described by Flügge in 1886 and was named *Bacillus oxytocus perniosus*. The term *Bacterium oxytocum* (Flügge) was later introduced by Migula in 1900. *B. oxytocum* was first isolated from stale milk and classified with *B. coli* and *B. lactis aerogenes* (Lautrop, 1956). MacConkey (1909) described this organism in considerable detail. It fermented all the sugars he used in his study, it was positive for indole production, non-motile and positive for the Voges-Proskauer test. It also liquefied gelatin slowly, taking as long as eight to twelve months to liquefy half an inch of the medium. Because this characteristic could not be readily used as a distinguishing feature, this organism could be incorrectly identified unless other differentiating tests were included.

Many researchers isolated klebsiella-type organisms from a variety of sources and described them as being distinct from, but related to the *Klebsiella* group. Constant reference to these organisms in the literature is evidence of the frequency of their occurrence. The difficulty associated with inclusion of *Oxytocum* strains in the *Klebsiella* group is well recorded. Some strains are indole and gelatin positive while others are indole positive and gelatin negative. Brooke (1951b) and Kauffman (1956) considered the indole and gelatin positive strains as belonging to the *Klebsiella* group, while Edwards and Fife (1955) included only the gelatin negative strains in the *Klebsiella* group. Ørskov *et al.* (1956) proposed that the

name *K. oxytoca* be used for indole and gelatin positive strains. However, *Klebsiella* capsule types were established for both gelatin positive and negative strains (Hugh, 1959).

Several authors suggested that *K. oxytoca* should be placed in a separate taxonomic unit, within the family Enterobacteriaceae (Cowan *et al.*, 1960; Hugh, 1959; Lautrop, 1956). This was also proposed by the Subcommittee for Enterobacteriaceae Taxonomy (Kaluzewski, 1967). Kaluzewski (1967) supported the separation of *K. oxytoca* into its own species within the genus *Klebsiella*. Based on his work and that of previous authors, Kaluzewski (1976) further concluded that

> ....the name *Klebsiella oxytoca* should be applied to all indole-producing strains which show other features typical for the genus *Klebsiella*, with no respect to their capability of gelatin liquefaction.

Contrary to this, Fife *et al.* (1979) considered that the differences between *K. oxytoca* and typical *Klebsiella* were insufficient to justify a separate species. They regarded it as a biotype of *K. pneumoniae*.

The 8th edition of Bergey's Manual (Buchanan & Gibbons, 1975) does not recognise *K. oxytoca* as a separate species, and considers it a biotype of *K. pneumoniae*.

The nomenclature of *K. pneumoniae* is still not resolved. Two separate systems are still in use. Workers in America and Western Europe refer to *K. pneumoniae (sensu lato)*, which includes *K. aerogenes/oxytoca/edwardsii/atlantae*. Workers in the United Kingdom use *K. pneumoniae*

(*sensu stricto*) to refer to Friedländer's bacillus, and *K. aerogenes* to refer to the *K. aerogenes/oxytoca/edwardsii/atlantae* group. This has undoubtedly led to confusion. Barr (1977) suggested that the U.K. adopt the usage of the term *K. pneumoniae* (*sensu lato*) to include *K. aerogenes/oxytoca/edwardsii/atlantae/pneumoniae*, and that they retain the name *K. pneumoniae* (*sensu stricto*) for Friedländer's bacillus.

As research progresses, *K. oxytoca* is becoming more accepted as a separate species. DNA-DNA hybridization (Jain *et al.*, 1974) and protein electrophoresis (Goullet, 1980) provided further evidence for the differentiation of *K. oxytoca* from *K. pneumoniae*.

In a numerical classification study of the tribe *Klebsiellae*, Bascomb *et al.* (1971) suggested the inclusion of *E. aerogenes* in the genus *Klebsiella*. They proposed the name *K. mobilis nomen novum*, to distinguish it from *K. aerogenes*. A new species, *K. terrigena*, isolated from unpolluted soil and drinking water, has been proposed by Izard *et al.* (1981), based on its phenotypic characters, protein electrophoretic patterns and DNA-DNA relatedness. They suggested the revision of the genus *Klebsiella* as follows: (i) *K. pneumoniae*, (ii) *K. ozaenae*, (iii) *K. rhinoscleromatis*, (iv) *K. oxytoca* (Flügge), (v) *K. mobilis*, and (vi) *K. terrigena*.

Serology

In an effort to resolve the taxonomic problem of the *Klebsiella* group, the serology of *Klebsiella* has also been investigated. The serology of the *Klebsiella* group is based on Toenniessen's differentiation of the bacterial cell into two components, the capsule and the soma (Brooke, 1951a). The capsular component is a carbohydrate that confers type specificity and virulence to the cell. If the capsule is removed, the organism loses its type specificity. The antigenic component of the soma is a species specific protein. Sera produced by a capsule-free Friedländer's bacillus react with all acapsular strains of the *Klebsiella* species (Julianelle, 1926a).

Julianelle (1926b) described two varieties of bacterial cells: smooth (S) forms characterized by mucoid growth in liquid media, capsule formation, increased virulence and type specificity; and rough (R) forms characterized by loss of capsule and mucoid characteristics, loss of type specificity, reduction of virulence and species specificity. S strains can be converted to R strains, and R colonies have rough and irregular surfaces.

Kauffman (1949) discovered that the serology of *Klebsiella* spp. involved four factors: the mucoid envelope, the capsule, the O antigen and the R antigen. Because of the similarity between the mucoid, acapsular and the non-mucoid capsular forms, he described only three of the factors: the capsular (K) antigen; the somatic, smooth (O) antigen; and

the somatic, rough (R) antigen. Kauffman (1949) also noted that the simple designations of S, R and M (mucoid) previously used by researchers, were insufficient to describe the different forms that were encountered. He suggested the following classification:

I. Smooth Forms

1. MKO = mucoid, capsular with O antigen
2. KO = non-mucoid, capsular with O antigen
3. MO = mucoid, acapsular, with O antigen
4. O = non-mucoid, acapsular with O antigen

II. Rough Forms

1. MKR = mucoid, capsular without O antigen
2. KR = non-mucoid, capsular, without O antigen
3. MR = mucoid, acapsular, without O antigen
4. R = non-mucoid, acapsular, without O antigen

The R antigen occurred in all smooth forms, but in the presence of fully developed O antigens, it was masked and gave only a weak antigenic reaction (Kauffman, 1949).

The formation of large, mucoid, moist colonies, as a consequence of prominent capsule formation, is a noticeable characteristic of the genus *Klebsiella* (Edwards & Ewing, 1972). Acapsular strains may be obtained by chemical or cultural methods. However, the use of these methods to isolate acapsular forms might produce rough variants of

changed antigenic constitution (Edwards & Fife, 1952). It would also strip the organisms of their type specificity. Therefore, for practical reasons, it is better to base serological identification on the recognition of capsular antigens. This is the only method in general use today (Edwards & Ewing, 1972). To date, 11 somatic O antigens (Buchanan & Gibbons, 1975) and 82 capsular K antigens have been reported. In reality, however, only 77 K antigens exist. Five were found to be identical or very closely related to types that already exist (Ørskov & Fife-Ashbury, 1977).

Investigations dealing with nosocomial *Klebsiella* infections have relied greatly on serotyping to identify *Klebsiella* strains. However, cross reactions between strains interfere with the efficiency of this method (Rennie & Duncan, 1974). They have suggested that the combined use of biotyping and serotyping is more helpful in subdividing *Klebsiella* strains into many more distinct types than either system used alone.

Habitat and Pathogenicity

1. *Clinical Isolates*

K. pneumoniae occurs normally in the intestines of 20 percent of healthy humans and 30 to 40 percent of healthy warm blooded animals (Bagley & Seidler, 1977; Campbell *et al.*, 1976). In the past, these organisms were rarely considered pathogenic; however, over the years, their

frequent association with clinical infection has made them a cause for concern (Ørskov, 1981).

K. pneumoniae is known to be responsible for 2 percent of all cases of acute bacterial pneumonia, with a 60 to 70 percent mortality rate attributed to antibiotic resistance (Caplenas *et al.*, 1981; Ørskov, 1981). In addition, it is an opportunistic pathogen, that causes a variety of secondary infections, especially in elderly, compromised or postoperative patients who are receiving antibiotic therapy (Ørskov, 1981). *Klebsiella* are most frequently isolated from human genito-urinary tract infections but they also cause many respiratory tract infections (Brown & Seidler, 1973; Caplenas *et al.*, 1981; Ørskov, 1981; Seidler *et al.*, 1975). Diseases caused by *K. pneumoniae* also include bacteraemia, osteomyelitis and meningitis (Caplenas *et al.*, 1981). A large percentage of the bacteraemiae may originate from infections of the urinary tract and such cases are often fatal (Ørskov, 1981). Enterotoxigenic *Klebsiella* may lead to outbreaks of diarrhoea in children. Newborn babies are extremely susceptible to colonization by *Klebsiella*, because there is little microbial competition in the intestine (Deb *et al.*, 1980). In domestic animals, *Klebsiella* has been identified as the primary causative agent of bovine coliform mastitis (Bagley & Seidler, 1977) and it is recognised as a serious and lethal agent of diseases (Knittel *et al.*, 1977).

Multiple resistant (MR) strains of *K. pneumoniae* were isolated from hospital patients with resistance to

gentamicin, tobramycin, cephalothin, chloramphenicol and ampicillin (Gerding *et al.*, 1979). The large scale use of chemotherapeutics and other antibiotics selects for resistant organisms that may carry R plasmids with drug resistant genes. Such plasmids may be transferred not only among strains of the same species, but also to other species (Ørskov, 1981). This has been demonstrated with the transfer of R plasmids from MR *K. pneumoniae* to *E. coli* K-12 (Gerding *et al.*, 1979). They observed that even when the incidence of new cases of MR *Klebsiella* infection was reduced, the rate of isolation of non-*Klebsiella* antibiotic resistant Gram negative bacilli had increased. This suggested that the transfer of R plasmids from MR *Klebsiella* was responsible for the increased isolation of these organisms, which included the genera: *Enterobacter*, *Proteus* and *Serratia*.

The epidemiology of *K. pneumoniae* infections is not clearly understood. Many authors have proposed that nosocomial infections with *Klebsiella* occur through intestinal colonization by the organism, which in turn serves as an important reservoir for autoinfection and cross-infection (Caplenas *et al.*, 1981; Casewell & Phillips, 1978; Cooke *et al.*, 1979; Gerding *et al.*, 1979). Precolonization often precedes infection (Gerding *et al.*, 1979). Symptomless patients colonized with *K. pneumoniae* were four times more susceptible to nosocomial infections than patients who were not colonized (Caplenas *et al.*, 1981). Cooke *et al.* (1979) showed that approximately 50

percent of patients with *Klebsiella* infections carried the same serotype in the bowel.

The hospital kitchen, food leaving the kitchen and to a lesser extent the hands of the hospital personnel, have been implicated as sources of *Klebsiella* in the colonizing of the intestines and spread of the organisms among patients. Foods that have been implicated include: salads and cold meat (Casewell & Phillips, 1978; Cooke *et al.*, 1980) and icecream (Casewell & Phillips, 1978). *Klebsiella* are seldom isolated from hot foods, however. The contamination of foods prepared in hospital kitchens with *Klebsiella* spp. of different serotypes has been demonstrated by Casewell and Phillips (1978) and Cooke *et al.* (1980). They reported that the same *Klebsiella* serotypes that were isolated from contaminated food ingested by patients were also isolated from the patients' faeces after ingestion of the food. Furthermore, autoinfection of patients by their acquired intestinal *Klebsiella* flora was reported by Selden *et al.* (1971). They found that patients whose intestines became colonized with *Klebsiella* after admission to hospital were more frequently victims of later *Klebsiella* infections by the same serotypes as that found in the intestines. Patients who were carriers at the time of admission rarely developed infections with the same intestinal serotypes.

In contrast, Casewell and Phillips (1978) could not demonstrate autoinfection of any patient by intestinal strains. They found that most of the acquired *Klebsiella*

infections were attributable to cross-contamination of other patients on the ward. Hands of hospital personnel were implicated as a significant route of transmission. Casewell and Phillips (1977) reported that 17 percent of hands washed yielded *Klebsiella*. On four occasions, the serotypes isolated from hands of staff were identical with those colonizing or infecting patients on the ward. In contrast, Hart *et al.* (1981) reported only one instance in which the *Klebsiella* strain isolated from hands of a staff member was the same serotype as those causing infection on the ward. Their results indicated that these organisms did not become part of the resident flora on the hands. Patients who were bed-ridden and whose faeces contained the organisms were more likely to carry these organisms on their hands.

2. *Environmental Isolates*

Klebsiellae are not restricted to the intestinal environment of humans and warm blooded animals, they are very widely distributed in nature. However, in the past, there was little significance attached to their frequent isolation from the environment. Isolates with the indole, methyl red, Voges-Proskauer, citrate (IMViC) pattern of --++ of clinical origin were called *K. pneumoniae*, while similar isolates of environmental origin were known as "*Aerobacter*" (Seidler, 1981). The widespread occurrence of *K. pneumoniae* in large numbers in the environment has been reported by many researchers. Table 1 summarizes the environmental

sources of *K. pneumoniae*, together with the cell densities at which they were reported.

The origin of environmental contamination by *Klebsiellae* is unresolved. Seidler (1981) suggested that the organisms originate from the faeces of insects, rodents, other wild animals and birds. Small inocula proliferate rapidly, since the botanical environment can provide the required nutrients to support the growth of clinical, faecal and environmental *Klebsiella* isolates.

The public health significance of *Klebsiella* in the environment is debatable. The following arguments have been put forward:

A. *Klebsiella* are not always isolated in association with *E. coli*, the officially accepted indicator of faecal contamination (Campbell *et al.*, 1976; Knittel *et al.*, 1977). This has been attributed to several factors: (i) *K. pneumoniae* are better survivors than *E. coli* (Bagley & Seidler, 1977). For example, in raw drinking water, *Klebsiella* made up 40 percent and *E. coli* 60 percent of the faecal coliforms, while in treated water, 67 percent of the faecal coliforms were *K. pneumoniae* and only 4 percent were *E. coli*; (ii) *Klebsiella* spp. have unique nutritional characteristics which enable them to survive and multiply in the environment (Bagley & Seidler, 1977). Their ability to fix nitrogen may also play a role in their colonization of the botanical environment (Knittel *et al.*, 1977).

TABLE 1. *Distribution of* K. pneumoniae (sensu lato) *in the Environment*

Source	Cell Density cfu/g or ml	Reference
Vegetable peel	10^3/g	Brown and Seidler, 1973
Salads	10^1 - 10^7/g	Wright *et al.*, 1976
Pulp and paper mill effluents	10^6/ml 10^8/ml	Knittel *et al.*, 1977 Caplenas *et al.*, 1981
Potatoes and lettuce	10^3/g	Knittel *et al.*, 1977
Alfalfa and bean sprouts	10^6/g	Patterson and Woodburn, 1980
Lake water	137/ml	Niemela and Väätänen, 1982

B. The stipulation that the occurrence of *K. pneumoniae* in the environment may indicate a health hazard is based on its potential pathogenicity and its identification as a faecal coliform. Many microbiologists do not accept *K. pneumoniae* as a potential health hazard because they are reluctant to believe that the environment can contain disease causing organisms in such large numbers (Knittel *et al.*, 1977).

Some microbiologists doubt the significance and validity of a positive faecal coliform test (production of gas from lactose fermentation at 44-45.5±0.2°C in 48 h) caused by *K. pneumoniae* in the absence of *E. coli* (Bagley & Seidler, 1977). In defence of the significance of the faecal coliform test when applied to *Klebsiella*, Bagley and Seidler (1977) showed that among *Klebsiella* cultures of known pathogenic origin, there was a high incidence (85 percent) of faecal coliform positive reactions. Subsequent testing of environmental *Klebsiella* isolates showed that 16 percent were faecal coliform positive. The test therefore, indicated that environmental sources of *Klebsiella* identified as faecal coliforms would be true faecal coliforms and not false positives. Bagley and Seidler (1977) concluded that *K. pneumoniae*, when found alone, should be considered as valid a faecal coliform as *E. coli*. Concurrent isolation of *E. coli* and faecal coliform positive *Klebsiella* from the environment should indicate recent faecal contamination, while isolation of faecal coliform positive *Klebsiella* alone

should indicate faecal contamination at some point in time, either recent or much earlier.

3. *Comparison of Clinical and Environmental Isolates*

Comparative studies have been done on both clinical and environmental *Klebsiella* isolates in an attempt to clarify their public health significance in the environment. Studies on the biochemical properties, serology, antibiotic sensitivity, DNA relatedness, faecal coliform reaction, colonization of the environment by clinical strains and pathogenicity of the environmental strains have been reported.

a) Biochemical Properties

In the past, the coliform group of organisms was differentiated on the basis of four biochemical tests: indole production, methyl red test, Voges-Proskauer test and citrate as the sole source of carbon, known by the mnemonic, IMViC. *E. coli* biotype I is classically recognised by the IMViC reaction ++--, while the *Klebsiella-Enterobacter* group was recognised by a --++ reaction. *Klebsiella* isolates with an IMViC pattern --++ were from a variety of sources - clinical, faecal and environmental. However, further research has shown that some *Klebsiella* spp. cannot always be classified by this IMViC pattern. *Klebsiella* from the same habitat exhibit different IMViC patterns and there is no correlation between IMViC pattern and habitat (Seidler *et*

al., 1975). Researchers have found that the saprophytic *K. aerogenes* is methyl red negative (MR-) and Voges-Proskauer positive (VP+) while the pathogenic *K. pneumoniae* (*sensu stricto*) is MR+ and VP- (Campbell & Roth, 1975). A total of seven different IMViC patterns have been found among environmental *Klebsiella* isolates (Brown & Seidler, 1973), compared to three to four patterns among human and animal isolates (Seidler, 1981). Some common patterns that have been reported are --++, -+++, ++++, -+-+ (Brown & Seidler, 1973). They reported that 50 percent of the environmental and human *K. pneumoniae* were --++, 28 percent were indole positive and 36 percent were MR+. The IMViC reaction -+-+ is uncommon among *Klebsiella* spp. It was previously used to identify another species, *Citrobacter freundii* (Campbell & Roth, 1975). However, it has been reported by many authors for the ATCC strain of *K. pneumoniae* #13883 (Brown & Seidler, 1973; Cowan *et al.*, 1960; Naemura *et al.*, 1979).

In other biochemical reactions, including the fermentation of various sugars, urease activity and decarboxylase and dihydrolase activities, no significant differences were found that could be used to distinguish between clinical and environmental isolates (Brown & Seidler, 1973; Matsen *et al.*, 1974; Seidler *et al.*, 1975). The single most variable reaction was the production of indole. Although indole positive strains (*K. oxytoca*) were isolated from both clinical and environmental sources (Woodward *et al.*, 1979), their frequency of isolation from

these sources varied among researchers. Edmondson *et al.* (1980) and Matsen *et al.* (1974) reported the occurrence of a higher proportion of indole positive strains among clinical isolates. In contrast, Bagley and Seidler (1977) reported that only 15 percent of clinical isolates were indole positive compared to 33 percent of environmental isolates, while Seidler *et al.* (1975) reported that 100 percent of *Klebsiella* isolates from living fir trees were indole positive compared to 17 percent of human clinical isolates. The isolates from the fir trees were among the most atypical strains encountered; some gave a weak H_2S reaction. Differences in urease activity were also reported (Bagley & Seidler, 1977): 100 percent of clinical isolates were urease positive compared to 75 percent of environmental isolates.

b) Serology

Serological studies have shown that human and environmental isolates are indistinguishable (Seidler *et al.*, 1975). *Klebsiella* strains isolated from infections, faeces, mastitis, hospital kitchens, water and botanical environments displayed a wide range of serotypes, with no one type or types predominating among isolates (Edmondson *et al.*, 1980).

c) Antibiotic Sensitivity

The widespread use of sulphonamides and other antibiotics contributed greatly to the reduction in

mortality rate of many bacterial infections. However, in the process, strains of organisms evolved with resistance to certain antibiotics. Some strains were resistant to drugs to which they had never been exposed. This was further complicated by the appearance of multiple-drug resistant strains (Falkow, 1975). Studies with members of the family Enterobacteriaceae showed that drug resistance was transmissible within and between species and the term resistance factor (R-factor) was adapted for this property (Falkow, 1975).

Selden *et al.* (1971) observed that the initial intestinal flora in patients who subsequently developed nosocomial infections was sensitive to all antibiotics tested (chloramphenicol, sulphonamides, kanamycin, streptomycin, neomycin, tetracycline) with the exception of ampicillin. After infection and antibiotic therapy, *Klebsiella* were isolated that were resistant to three and up to five of the antibiotics. Knittel *et al.* (1977) found transmissible R-factors among isolates from the botanical environment and suggested that plant material may serve as a reservoir for the spread of known pathogenic strains.

Comparative studies by Edmondson *et al.* (1980) on the extent of antibiotic resistance of *Klebsiella* strains showed the following results: clinical isolates and isolates from mastitis and the faeces of patients were most resistant, being resistant to five or more antibiotics, while strains isolated from the hospital environment, including the

kitchen and food were less resistant. The least resistant strains were those isolated from the faeces of outpatients with no known infections, flowers from both inside and outside the hospital and from river water. The majority of the least resistant isolates (more than 70 percent) were sensitive to eight or more antibiotics. The same trend was observed by Matsen *et al.* (1974) who reported that *Klebsiella* isolates from natural receiving waters were more susceptible to antibiotics than human clinical isolates. Strains from all sources were found to be highly resistant to ampicillin and carbenicillin (Edmondson *et al.*, 1980; Matsen *et al.*, 1974). Matsen *et al.* (1974) also observed that the greatest number of strains susceptible to ampicillin (27 percent) and carbenicillin (12 percent) was isolated from clean saline water sources.

d) DNA Relatedness

Although *Klebsiella* isolates of both clinical and environmental origin are phenotypically indistinguishable, DNA reassociation studies have shown that environmental isolates are genetically more diverse than human clinical isolates. Seidler *et al.* (1975) observed a range of 5 to 100 percent in relative DNA reassociation. They examined *Klebsiella* strains from four different environments: human, river, vegetable and pulp mill effluents. The vegetable isolates showed the greatest diversity from the human reference strain, *K. pneumoniae* ATCC 13883. The average

homology was only 35 percent. Most of the human isolates showed an average homology of greater than or equal to 75 percent, with comparable degrees of homology occurring between the reference organism and the river and pulp mill isolates.

Similar observations were made by Woodward *et al.* (1979). Unlike Seidler *et al.* (1975), they used the faecal coliform strain of *K. pneumoniae* ATCC 13882 as the reference strain. However, they reported that it was genetically indistinguishable from the non-faecal coliform strain ATCC 13883 and had the IMViC pattern --++. Both were originally human isolates. Based on relative reassociation studies they concluded that although the majority of environmental *Klebsiella* was of the same phenotype as *K. pneumoniae* (*sensu stricto*), they were genetically distinct, and as such were not all members of this species. In fact, they formed a molecular group among themselves that Woodward *et al.* (1979) considered should be three new taxa. However, these genetically diverse *Klebsiella* spp. were capable of causing clinical infections, since three strains had been isolated from clinical material.

e) Faecal Coliform Reaction

The faecal coliform test (growth and gas production from lactose at 44-45.5°C) is normally used to define the origin of a coliform (ICMSF, 1978; Naemura & Seidler, 1978). Naemura and Seidler (1978) reported that eighty-five percent

of *Klebsiella* cultures of known pathogenic origin were faecal coliforms. However, 16 percent of environmental isolates were also faecal coliforms (Bagley & Seidler, 1977). It was suggested that the contamination of fruits and vegetables by faecal coliforms may be through insects, direct defaecation of wild and farm animals, polluted irrigation water and use of manure (Geldreich & Bordner, 1971).

Naemura and Seidler (1978) postulated that environmental isolates might have lower minimum growth temperatures than clinical isolates. They found an inverse relationship between faecal coliform positive isolates and growth at 10°C, regardless of habitat. One exception was the non-faecal coliform strain, ATCC 13883, which did not grow at 10°C. Since failure to grow at 10°C is a characteristic of faecal coliforms, it was suggested that *K. pneumoniae* ATCC 13883 may have been a faecal coliform that mutated to a non-faecal coliform (Naemura & Seidler, 1978). It was noted that many of the non-faecal coliform isolates that grew at 10°C also grew anaerogenically at 44.5°C. They have been termed "thermotolerant". Other comparisons made between faecal and non-faecal coliforms were:

1. All faecal coliform isolates were indole negative (Naemura & Seidler, 1978). Indole positive strains (70 percent) were reported among non-faecal, clinical isolates (Edmondson *et al.*, 1980). Because of the clinical source of these indole positive, non-faecal coliform strains, it was

suggested that they may be more significant in causing infections than other indole positive non-faecal strains that were not clinical isolates (Edmondson *et al.*, 1980).

2. Faecal coliform strains were more resistant to antibiotics than non-faecal coliforms, regardless of indole reaction.

f) Colonization of the Environment

The observation that environmentally derived *Klebsiella* were genetically more diverse than clinical isolates raised questions about the ability of clinical isolates to colonize the environment and their ability to compete with the indigenous flora (Knittel *et al.*, 1977). Pathogenic and environmentally derived *Klebsiella* isolates survived equally well in filter sterilized and raw pulp wastes and on the surfaces of potatoes and lettuce, reaching cell densities of 10^6/ml in the pulp wastes and 10^3/g on the vegetables within 24 hours. The counts were comparable to those of indigenous *Klebsiella* of unknown origin. This study also provided evidence that *Klebsiella* isolates were able to colonize sawdust bedding and that they could spread from cow to cow, infecting the teats and causing mastitis.

The indirect contamination of lettuce by first immersing hands in different *Klebsiella* suspensions (10, 10^2, 10^3 cfu/ml), showed that *Klebsiella* grew on the lettuce even at the lowest levels of hand contamination. This implicated hands as a source of vegetable contamination

(Knittel *et al.*, 1977). Similar studies by Yamamoto and Seidler (1981) with radish seeds showed that, regardless of the initial inoculum (10^0 to 10^4/seed), the *Klebsiella* count of both clinical and environmental isolates reached 10^7 cfu/g of plant material after the first week. The counts decreased weekly as the plants grew, but even after five weeks, counts were still in the range of 10^4 to 10^5 cfu/g of plant material. The *Klebsiella* organisms were not found inside the plant or on the leaves, but on the root bulb surface and the attached soil. Furthermore, *Klebsiella* survived well on plants, but when the soil alone was inoculated with similar concentrations of *Klebsiella* as the radish seeds, the organisms failed to grow.

Survival of *Klebsiella* isolates inoculated onto radish seeds varied with source of the isolate, in the following decreasing order of survival: vegetables, mastitis, human infection, water, pulp mill effluent. Nevertheless, contamination with isolates from all sources, resulted in a count of greater than 10^3 cfu/g of plant material at time of harvest. There was no correlation between survival and faecal coliform reaction or density of initial inoculum (Yamamoto & Seidler, 1981).

The effect of repeated growth of pathogenic *Klebsiella* in the botanical environment on the virulence of these organisms was studied by Knittel *et al.* (1977). *Klebsiella* from human infections and bovine mastitis were passaged through pulp wastes for a period of seven weeks. Changes in

virulence were monitored in mice by determining the LD_{50} values. Isolates from bovine mastitis did not decrease in virulence despite about 290 generations of growth. The virulence of human isolates decreased significantly but only after about 100 generations of growth. Knittel *et al.* (1977) concluded that plant material could serve as a reservoir for the spread of pathogenic *Klebsiella*.

g) Pathogenicity

Studies using LD_{50} techniques were done on mice to determine the potential pathogenicity of environmental isolates of *K. pneumoniae* compared to clinical isolates of known pathogenicity (Bagley & Seidler, 1978b; Matsen *et al.*, 1974). Matsen *et al.* (1974) reported a mean LD_{50} value for mice of 4.5×10^5 cells/ml for water isolates compared to 5.3×10^5 cells/ml for human clinical isolates, indicating virtually no difference in virulence between the two groups. Similarly, Bagley and Seidler (1978b) reported no significant difference in the mean LD_{50} values for mice for *Klebsiella* isolated from different environments. In contrast, they found that *E. coli* isolates from drinking water had a significantly higher mean LD_{50} value than clinical *E. coli* and all of the *Klebsiella* isolates. These results indicated that *K. pneumoniae* isolates from any source, were equally pathogenic for mice. This included faecal and nonfaecal *Klebsiella* and *K. oxytoca*.

Bagley and Seidler (1978b) concluded that *Klebsiella* from environmental sources have a dual role: (1) they are opportunistic pathogens for humans and animals, and (2) faecal coliform positive strains serve as indicators of faecal contamination. Since faecal coliform negative strains and *K. oxytoca* are as pathogenic as faecal coliform positive strains, they should also be considered a potential health hazard (Bagley & Seidler, 1978b).

B. Effects of Low Temperature Storage on Bacteria

In the food industry, freezing is an important method for preserving or prolonging the shelf-life of foods (Calcott, 1978). Bacterial cells that survive freezing may do so with no apparent adverse effect or they may be sublethally injured. The judgement of these different states of viability is based on the ability of the cells to multiply and form colonies on different types of solid media (Ray & Speck, 1973). Uninjured cells are those that multiply and form colonies on nonselective and selective agar media. Nonlethally injured cells are of two types:

(a) metabolically injured - those able to multiply and form colonies on nonselective media (for example, tryptic soy agar) but not on minimal salts media;

(b) structurally injured - those able to multiply and form colonies on nonselective media, but not on selective media (for example, violet red bile agar).

The different states in which bacterial cells exist in frozen foods can complicate the detection of pathogens and indicator organisms. It is important, therefore, that special methods should be used to allow the repair (resuscitation) of nonlethally injured cells. Without these techniques, frozen foods could be an unsuspected health hazard (Ray & Speck, 1973; Speck *et al.*, 1975). Incubation of the injured cells in nonselective media restores their ability to grow on selective media (Mackey *et al.*, 1980).

The susceptibility of bacterial cells to freezing damage differs between strains and species of microorganisms, the age of the culture, the nature of the medium in which the cells are suspended and the conditions of freezing and thawing (Ray & Speck, 1973).

Gram positive cells are generally more resistant to damage by freezing than Gram negative cells (Calcott, 1978; Ray & Speck, 1973; Speck & Ray, 1977). Williams *et al.* (1980) reported significant reductions in coliform and *E. coli* from ground beef stored at -20°C for 4 days, while significant reductions in *Staphylococcus aureus* occurred only after 11 days of storage at -20°C. The reason for this difference between Gram positive and Gram negative cells is not known, but it may be due to the less complex nature of the Gram positive cell envelope (Calcott, 1978). It is possible that the lipopolysaccharide layer that forms the permeability barrier of Gram negative cells is damaged during freezing and thawing, allowing leakage of cellular

materials and rendering the cells more susceptible to solutes and toxic substances in their environment (Ray & Speck, 1973).

Studies have generally shown an inverse relationship between bacterial survival and temperature. It was found that at temperatures above -80°C, the death rate was greater, while below -80°C, bacteria exhibited considerably reduced death rates (Calcott, 1978). Straka and Stokes (1959) observed that the death rate of *Pseudomonas fluorescens* was slowest at -29°C and fastest at -7°C. Death and/or injury can occur even at temperatures of 0°C and above. Patterson and Jackson (1979) reported that the viability of *E. coli* and *S. aureus* in the exponential growth phase decreased if these organisms were stored at 1 and 4°C. Similar results were obtained with *Vibrio vulnificus* held at 4°C (Oliver, 1981) and *Aerobacter aerogenes* at 0°C (Strange & Dark, 1962; Strange & Ness, 1963).

Death or injury of bacterial cells chilled from their optimum growth temperatures to 4 to 0°C without actual freezing, is a phenomenon known as cold shock (Calcott, 1978; Oliver, 1981). Several factors have been implicated in survival of bacteria during cold shock, including: growth temperature, growth phase, ionic strength and species of salts in the cell diluent, concentration of cells, rate of chilling, period of storage, plating medium and repair of damage caused by cold shock (Oliver, 1981). Bacterial cells are only susceptible to cold shock when they are in the

exponential phase of growth, and injury only occurs when cells are chilled rapidly (Calcott, 1978; Strange & Dark, 1962). Injury results from apparent damage to the permeability barriers of the cell membrane and cell wall (Calcott, 1978).

In general, bacterial cells in the exponential growth phase are more susceptible to injury at low storage temperatures than cells in the stationary phase (Calcott, 1978; Patterson & Jackson, 1979; Postgate & Hunter, 1961). This difference in susceptibility is probably caused by the fact that cells in the exponential phase are multiplying and their structural and functional macromolecules are being synthesised, assembled or reorganised. Consequently, they are more susceptible to damaging conditions. On the other hand, cells in the stationary phase are more stable and therefore not as vulnerable (Ray & Speck, 1973).

The rates of freezing and thawing markedly affect survival of bacterial cells. Less damage occurs with a slow rate of freezing and a rapid rate of thawing (Calcott, 1978; Postgate & Hunter, 1961; Ray & Speck, 1973). Repeated freezing and thawing increases bacterial death, while the percentage of nonlethally injured cells among the survivors remains constant (Ray & Speck, 1973).

Survival of bacterial cells during freezing and frozen storage may also be affected by pH. Lactic streptococci survived freezing and frozen storage better at pH 7.0 than at pH 5.0. *E. coli* and *P. fluorescens* frozen in meat extract

between pH 5.0 and 8.0 survived better between pH 6.0 and 7.0 than at either of the extremes. This was more pronounced at higher temperatures of frozen storage (-7°C) than at lower temperatures (-30°C) (Ray & Speck, 1973).

The length of storage time of bacterial cultures is directly related to the amount of death that occurs (Ray & Speck, 1973). Straka and Stokes (1959) observed that death of cultures of *P. ovalis*, increased, over a 19-day period, from 22 percent to 53 percent.

The nature of the suspending medium is an important factor in survival or death of the cells. Distilled water and NaCl were reported to be highly lethal in the freezing and thawing of bacterial suspensions. Addition of glycerol to the suspending medium gave some protection to the cells (Postgate & Hunter, 1961). Phosphate added with NaCl to the suspending medium gave some protection to cultures of *E. coli* and *A. aerogenes* (Ray & Speck, 1973). *A. aerogenes* grown in a defined medium was highly susceptible to chilling, but it was more resistant when grown in a tryptic meat broth (Strange and Ness, 1963). In contrast, Postgate and Hunter (1961) reported extensive death of *A. aerogenes* cultures that were frozen in tryptic meat broth. *E. coli*, however, was found to be almost completely resistant to the effects of chilling in both the defined medium and the tryptic meat broth (Strange & Ness, 1963).

This protective effect in some media is attributed to cryoprotective agents in the suspending medium. Frozen meat

contains cryoprotective agents, which include proteins, simple and complex carbohydrates and triglycerides (Mackey *et al.*, 1980). Meat also contains compounds which reduce the resistance of bacterial cells to freezing. These include ions, inorganic salts, acids, surface active components and certain enzymes such as lysozyme and proteases (Speck & Ray, 1977). Some proteolytic and nucleolytic enzymes which are usually present in the bacterial cell in a latent form (Calcott, 1978), are activated during freezing and storage (Ray & Speck, 1973).

The mechanism of the protective effect against freezing death is not clear, however, hydrogen bonding was reported to be important (Calcott, 1978). Protection against damage from frozen storage occurs either by eliminating the action of toxic substances from the external environment or by increasing the resistance of the sites on the bacterial cell that are sensitive to the toxic agents. Similarly, the mechanism of injury caused by cold and frozen storage is not clear. It is believed that injury and death are the result of ice crystal formation internally or externally depending on the rate of freezing and the length of storage. If the rate of freezing is rapid, internal freezing of the water in the cell takes place and small, unstable ice crystals are formed. Recrystallization of these ice crystals due to fluctuations in temperature during frozen storage causes death of bacterial cells. The ice crystals that are formed destroy the membrane components of the cell. If the rate of

freezing is slow, all extractable water is removed from the cell cytoplasm and freezes externally. Thus, damage is caused by dehydration (Calcott, 1978). Death by frozen storage may result from prolonged exposure to the extracellular and intracellular solutes which become concentrated when ice separates during freezing. This is called osmotic shock.

Bacterial cells are not lysed by freezing and thawing, but there is marked leakage of cellular material. The materials that leak from the cells include low molecular weight bases and nucleotides, amino acids and small peptides (Calcott, 1978; Moss & Speck, 1966). Peptides released are biologically active. They protect the cells from the lethal effects of freezing as well as aiding them in the repair of nonlethal injury (Moss & Speck, 1966). Compounds leaked from frozen cells may be different to those leaked from rapidly chilled cells. The viability of bacterial cells is related to the nature of the leakage products, rather than the amount of leakage that occurs.

Freezing and thawing are not mutagenic, but frozen bacterial cells show increased sensitivity to the effects of ultra-violet light and to sunlight. The rate of mutation is increased. Sensitivity to UV light is more pronounced in the presence of oxygen (Calcott, 1978).

IV. MATERIALS AND METHODS

Bacterial Cultures

Five bacterial cultures were used in this study. Three were freeze-dried cultures from the American Type Culture Collection (ATCC): *Escherichia coli* ATCC 11775, *Klebsiella pneumoniae* ATCC 13883 and *K. oxytoca* ATCC 13182; and two cultures were previously isolated from meat: *K. pneumoniae* #2 and *K. oxytoca* #1765.

All five cultures were maintained on nutrient agar slants and stored at 4°C. Once every month the cultures were checked for purity by growing three successive subcultures in nutrient broth and then streaking them onto tryptic soy agar plates. A colony from each culture was used to inoculate a fresh nutrient agar slant and their biochemical characteristics were checked using the BBL Minitek enteric system incubated at 35°C for 18 h, with the following biochemical discs: nitrate without dextrose, phenylalanine, hydrogen sulfide/indole, Voges-Proskauer, citrate, ONPG, urea, lysine, arginine, ornithine, dextrose, malonate, adonitol, arabinose, inositol, raffinose, sorbitol, lactose, rhamnose, sucrose (BBL, Becton-Dickinson Mississauga, Canada).

Preparation of Cultures

Various characteristics of the bacterial cultures were studied including: coliform reaction, ability to produce

indole from tryptophan, ability to ferment glucose, lactose and sucrose on triple sugar iron (TSI) agar slants, and growth rates. In addition, the effects of low temperature storage on suspensions of pure cultures and on cultures inoculated into ground beef of normal pH (5.5) and higher pH levels were studied.

Coliform and Indole Tests. All five stock cultures were tested for coliform and faecal coliform reaction. The cultures were inoculated into EC broth (Difco) containing inverted Durham tubes and incubated at 35 and 44.5°C for 48 h. Those organisms that failed to grow or produce gas at 44.5°C were tested at lower temperatures, to determine the maximum temperature at which gas production occurred. Similar studies were conducted for indole production from tryptophan by *E. coli* and the *K. oxytoca* isolates. Tubes of tryptic soy broth (Difco) were inoculated and incubated at 35°C and at elevated temperatures for 48 h to determine the maximum temperature at which indole was produced. Kovac's reagent was used to detect indole production (MacFaddin, 1981).

Determination of Growth Curves. Throughout this study, the test cultures were used in the late log, early stationary phase of growth. Growth curves were determined in nutrient broth (NB, Difco) at 35°C in a shaking incubator (New Brunswick Scientific Co., Inc.) at 200 rpm. A 1 ml aliquot

of an 18 to 24 h culture of the test organism was added to 99 ml of NB in an Erlenmeyer flask and incubated as stated above. Samples were taken at 30 min intervals for 7 h. Samples were serially diluted in 99 ml of 0.1 percent (w/v) peptone water blanks and surface plated in triplicate onto standard plate count agar (SPC, Difco). The SPC plates were incubated at 35°C for 18 to 24 h before counting. The increase in the log number of cfu/ml with time was plotted, and a time interval in the log phase of growth was selected for determining the generation time.

Experiments with Test Cultures Stored in Different Broths

Four different broths were used in this study: tryptic soy broth (TSB, Difco), one-tenth the normal strength of TSB (10%TSB), NB and Cooked Meat Medium. Five ml of each broth were inoculated with 0.05 ml of culture to give approximately 10^6 to 10^7 cfu/ml. In the case of cooked meat medium, 10 ml were inoculated to facilitate pipetting. The inoculated broths were incubated at 35°C for 6 h and stored at 10, 4, and -16±2°C (commercial grade of freezer) for 7 days. Initial samples were taken before storage (day 0) and on days 3 and 7. Separate samples were used for analysis on each of the different days. Samples were serially diluted in 99 ml of 0.85 percent (w/v) saline dilution blanks. Here, and in the experiments following, saline dilution blanks were used instead of peptone blanks to avoid any influence that the peptone might have on the survival of the test

cultures. Frozen samples were thawed by holding for approximately 3 min under lukewarm (22°C) flowing water. A 0.1 ml aliquot of appropriate dilutions was surface plated in triplicate onto prepoured selective and nonselective media (Difco) including tryptic soy agar (TSA), violet red bile agar (VRBA) with a 5 ml VRBA overlay, TSA incubated for 2 h at 35°C for repair of the cells, then overlayered with 12 ml VRBA (TSA/VRBA) (Speck *et al.*, 1975) and tryptone bile agar (TBA) (Anderson & Baird-Parker, 1975). The plates were incubated at 35°C and enumerated after 18 to 24 h.

TSA was used to measure the total number of survivors. Three to 5 colonies were selected from the TSA plates for further biochemical studies. These colonies were selected on the basis of different morphological appearance (size) or at random. VRBA was used to measure the number of uninjured survivors. Typical colonies are purplish-red in colour and produce a ring of bile precipitate. Three to 5 colonies were selected for further biochemical studies based on size and/or the degree of bile precipitation. TSA/VRBA was used to measure the number of injured cells capable of repair. The inoculum was plated onto TSA and incubated at 35°C for 2 h after which it was overlayered with 12 ml of VRBA and incubated at 35°C for 18 to 24 h. Colonies on this medium are purplish-red in colour and have a faint bile precipitate. Three to 5 colonies were selected on the basis of size or at random for further biochemical studies. TBA was used to screen cells for indole production. This medium

has the following composition: Tryptone 2.0 g, Bile Salts 0.15 g, Agar 1.5 g, 100 ml distilled water.

The procedure proposed by Anderson and Baird-Parker (1975) was modified by plating directly onto TBA instead of using cellulose acetate filter membranes (Bueschkens, 1982). Inoculated plates were incubated at 35°C for 18 to 24 h. Colonies grown onto the TBA plates were replica-plated onto new TBA plates using sterile 12.5 cm Whatman #1 filter paper. Both the original and the replica plates were incubated at 35°C for 24 h. After incubation, the original plates were flooded with TBA reagent of the following composition: 2.0 g *p*-dimethylaminobenzaldehyde, 92.0 ml distilled water, 8.0 ml concentrated HCl. After approximately 1 minute, the excess reagent was drained off and the plates were examined against a black background. Colonies that were indole positive were stained reddish-pink in colour, while indole negative colonies were stained a white-yellow colour. For satisfactory results, it was necessary that the colonies on the plates were well separated. Any indole negative colonies were selected from the corresponding replica plates for further biochemical studies.

Selected biochemical characteristics that distinguish *E. coli* from *K. pneumoniae* (*sensu lato*) and *K. pneumoniae* from *Enterobacter* spp., were examined for any changes caused by low temperature storage. The following characteristics were studied: glucose, lactose and sucrose fermentation, as

well as H_2S production on TSI agar slants, motility on motility agar (MacFaddin, 1981), growth in EC broth with gas production at 35 and 44.5°C and indole production in tryptic soy broth at 35 and 44.5°C. All tests were incubated at 35°C for 24 h, unless elevated temperature was also indicated.

Experiments with Test Cultures Stored in Raw Ground Beef

A 0.1 ml aliquot of an 18-24 h culture of the test organism in NB was subcultured into 10 ml NB and incubated at 35°C for 6 h to give a cell density of 10^8 to 10^9 cfu/ml. The cells were centrifuged at 1000 x g for 10 min in a centrifuge (Heraeus Christ Labofuge 6000), washed once with 1 ml of 0.85 percent saline solution and resuspended in 1 ml for use in the experiment.

Approximately 250 g of fresh, commercially prepared ground beef (30 percent fat) was divided into 10-g portions. A set of uninoculated 10-g samples was prepared as controls. Each of the remaining 10-g samples was inoculated with 0.1 ml of a prepared cell suspension to give 10^7 to 10^8 cfu/g of ground beef. The inoculum was mixed thoroughly into each sample, using a sterile spatula. All samples were placed in separate bags for storage. The inoculated and uninoculated samples were divided into 3 groups for storage at 4, -16±2°, and -40°C (biological storage freezer, Forma Scientific) for up to 7 days. Samples stored at 4°C were sampled on 0, 1, 2, 3 and 7 days of storage. Samples stored at -16 and -40°C were sampled on 0, 3 and 7 days of storage.

The inoculated and uninoculated ground beef samples from each of the three storage temperatures were blended with 90 ml of 0.85 percent saline in a Colworth Stomacher Lab-Blender 400. Serial dilutions of each sample were prepared and appropriate dilutions were inoculated in duplicate onto the following selective and nonselective media: TSA, VRBA, TSA/VRBA, as described for the previous experiment, and onto TBA for *E. coli* and *K. oxytoca* organisms, and MacConkey Inositol Carbenicillin (MCIC) agar (Bagley & Seidler, 1978a) for *K. pneumoniae* and *K. oxytoca*. MCIC consists of 40 g MacConkey agar base, 10 g inositol, in 1000 ml distilled water. After autoclaving and cooling to 45°C, 0.5 g carbenicillin ("Pyopen", Ayerst Laboratories, Montreal, Canada) was added to the medium. Its specificity is based on the ability of virtually all *Klebsiella* cultures to ferment inositol and their high resistance to the antibiotic, carbenicillin. Plates were surface spread and incubated at 35°C for 18 to 24 h. *Klebsiella* cultures on this medium are a pink colour. Colonies were selected from the various plates and checked for phenotypic variants as described earlier for the broth cultures. The pH of the meat was also recorded on each day. All analyses were done in triplicate.

Effect of pH on Survival of Test Cultures Stored in Ground Beef and Broths

For inoculation of the ground beef, 25 ml of NB in a 125 ml Erlenmeyer flask was inoculated with 0.25 ml of an 18 to 24 h culture and incubated at 35°C for 6 h. Approximately 1.25 kg of fresh, commercially prepared ground beef (30 percent fat) was divided into three 200-g portions, and two 250-g portions. An 11-g sample was kept uninoculated from each portion as a control, and one of each of the three 200-g portions was adjusted to pH 5.5, 6.0 and 6.5 using 1N NH_4OH. Each portion was subdivided into 60-g portions, each of which was inoculated with 0.6 ml of prepared culture. The inoculum was mixed thoroughly into each portion using a food mixer. The 60-g portions were further subdivided into 11-g samples and placed in separate stomacher bags for storage. Inoculated and uninoculated samples were stored at 4°C for up to 3 days.

One of the 250-g portions was vacuum packaged, (degree of vacuum, 0.90 atm.) using a Multivac AG500; the other was stored in an aerobic package, and both held at 10°C for 24 h before further treatment. After 24 h, 11-g samples were taken as uninoculated controls, and the rest of the 250-g portion was inoculated with 0.6 ml of inoculum as described above. Each portion was subdivided into 11-g samples for storage in stomacher bags at 4°C for up to 3 days. Samples were taken for analysis initially (day 0) and after 1, 2 and 3 days of storage.

Each day, inoculated and uninoculated samples were blended with 99 ml of 0.85 percent saline in a Colworth Stomacher Lab-Blender 400. Serial dilutions of each sample were prepared using 99 ml dilution blanks and appropriate dilutions were inoculated in duplicate onto the following media: TSA/VRBA and VRBA, for *E. coli* cultures; and TSA/VRBA and MCIC for *K. pneumoniae* and *K. oxytoca* cultures. Plates were incubated at 35°C for 18-24 h. The pH of all samples was recorded on each day.

For broth inoculation, 10 ml of NB was inoculated with 0.1 ml of an 18-24 h culture and incubated at 35°C for 6 h. Tubes containing 10 ml of TSB or NB were adjusted to pH 5.5, 6.0 and 6.5 with 1N NaOH. All tubes were inoculated with 0.1 ml of the prepared culture and stored at 4°C for up to 3 days. Samples were taken initially (day 0) and after 1, 2 and 3 days of storage. Samples were serially diluted in 99 ml dilution blanks and appropriate dilutions were inoculated in duplicate onto media as described above for the beef samples. The pH of all samples was recorded on each day. All analyses were done in triplicate.

V. RESULTS

Minitek and IMViC Results

The biochemical characteristics of the five test cultures were determined using the BBL Minitek enteric identification system. The results for the biochemical data are shown in Table 2. The biochemical characteristics of the test cultures were monitored throughout the study. No changes in biochemical characteristics of the stock cultures were recorded.

The IMViC (Indole production, Methyl red, Voges-Proskauer and Citrate as the sole source of carbon) reactions were also determined for each of the test cultures. The results are shown in Table 3. The atypical IMViC reaction for *K. pneumoniae* ATCC 13883 has also been reported by other researchers (Bagley & Seidler, 1977).

Coliform and Indole Tests

The results for the coliform and indole tests are shown in Table 4. All cultures produced gas in EC broth at 35°C within 48 h. Only *E. coli* ATCC 11775 and *K. pneumoniae* #2 were faecal coliforms, capable of producing gas in EC broth at 44.5°C within 48 h. *K. pneumoniae* ATCC 13883, *K. oxytoca* ATCC 13182 and *K. oxytoca* #1765 varied in the maximum temperature at which cultures could grow and produce gas in lactose (EC) medium. They produced gas at 38, 42 and 43°C, respectively. *E. coli* ATCC 11775 produced indole from

TABLE 2. *Biochemical Reactions of the Test Cultures Based on the Minitek Enteric Identification System*

Biochemical Discs	Test Cultures[1]				
	1	2	3	4	5
Nitrate Reductase	+	+	+	+	+
Phenylalanine	-	-	-	-	-
H_2S	-	-	-	-	-
Indole	+	-	-	+	+
Voges-Proskauer	-	-	+	+	+
Citrate	-	+	+	+	+
ONPG	+	+	+	+	+
Urea	-	+	+	+	+
Lysine	-	+	+	+	+
Arginine	-	-	-	-	-
Ornithine	+	-	-	-	-
Dextrose without Nitrate	+	+	+	+	+
Malonate	-	-	-	+	-
Adonitol	-	+	+	+	+
Arabinose	+	+	+	+	+
Inositol	-	+	+	+	+
Raffinose	-	+	+	+	+
Sorbitol	+	+	+	+	+
Lactose	+	+	+	+	+
Rhamnose	+	+	+	+	+
Sucrose	-	+	+	+	+

[1]Key: 1 = *E. coli* ATCC 11775; 2 = *K. pneumoniae* ATCC 13883;
3 = *K. pneumoniae* #2; 4 = *K. oxytoca* ATCC 13182;
5 = *K. oxytoca* #1765

TABLE 3. *IMViC Reactions of the Test Cultures*

Organisms	Indole	Methyl red	Voges-Proskauer	Citrate
E. coli ATCC 11775	+	+	-	-
K. pneumoniae ATCC 13883	-	+	-	+
K. pneumoniae #2	-	-	+	+
K. oxytoca ATCC 13182	+	-	+	+
K. oxytoca #1765	+	-	+	+

TABLE 4. *Maximum Temperatures for Gas Production from Lactose and Indole Production from Tryptophan by Test Cultures*

	Gas Production		Indole Production	
	35°C	Elevated Temperature 44.5°C	35°C	Elevated Temperature 44.5°C
E. coli ATCC 11775	+	+	+	+
K. pneumoniae ATCC 13883	+	-	-	-
K. pneumoniae #2	+	+	-	-
K. oxytoca ATCC 13182	+	-	+	+
K. oxytoca #1765	+	-	+	+

tryptophan up to 44.5°C. *K. oxytoca* ATCC 13182 and meat strain #1765 also produced indole up to 44.5°C.

Determination of Growth Curves

The growth rate constant and generation time at 35°C were calculated using the following equations:

$$\text{Growth rate constant} = k = \frac{\log_{10} n_2 - \log_{10} n_1}{0.301 \times t}$$

Generation time = 1/k

where: n_1 = number of cells at time t,

n_2 = number of cells at a later time,

t = length of time from n_1 to n_2 (minutes).

E. coli ATCC 11775 had a generation time of 28 min, *K. pneumoniae* ATCC 13883 and *K. oxytoca* ATCC 13182, 25 min and the two meat isolates, *K. pneumoniae* #2 and *K. oxytoca* #1765 had generation times of 17 and 20 min, respectively. All cultures reached late log, early stationary phase in 6 h, when grown in NB and had a cell density of 1×10^9 to 2×10^9 cfu/ml, with the exception of *K. pneumoniae* ATCC 13883 which had a cell density of 6×10^8 cfu/ml.

Survival of Test Cultures Stored in Different Broths

This experiment was a pilot study to determine the effect of time and temperature of storage on survival of the test cultures. *E. coli* ATCC 11775, *K. pneumoniae* ATCC 13883 and meat strain #2 and *K. oxytoca* #1765 were stored in

broths of differing nutrient composition (TSB, 10%TSB, NB and Cooked Meat Medium) at different storage temperatures (10, 4 and -16°C) for up to 7 days. Survival of the organisms was measured by plating onto nonselective (TSA), selective (VRBA) and "repair" media (TSA/VRBA). The data for the log number of survivors of each organism under each condition of storage are shown in Tables 5 to 8.

Storage temperature and suspending medium had a marked effect on survival of the test cultures. At 10 and 4°C, there was no marked decrease in cells. Neither was there any marked difference between storage at 10 and 4°C. At -16°C, however, there was marked death of the cells by as much as 4 log cycles. The test cultures generally survived better in TSB and Cooked Meat medium than in 10%TSB and NB. This was especially the case at -16°C.

Of the test cultures, *E. coli* 11775 generally survived better than *Klebsiella* at 10 and 4°C. The exception was *K. pneumoniae* #2 which survived better in Cooked Meat medium than *E. coli* 11775. The meat strains *K. pneumoniae* #2 and *K. oxytoca* #1765 survived better in Cooked Meat medium at -16°C than the ATCC strains, *E. coli* 11775 and *K. pneumoniae* 13883. *K. pneumoniae* ATCC 13883 was most sensitive to the storage conditions. Length of storage had a variable effect on survival of the different organisms at all storage temperatures. However, in general, the cultures remained stationary from the 3rd to the 7th day of storage.

TABLE 5. *Survival of* E. coli *ATCC 11775 Stored in Different Suspending Media at Various Temperatures*

Susp. Media	Plating Media	Initial No.	Survival of Cells (log cfu/ml) 10°C Day 3	10°C Day 7	4°C Day 3	4°C Day 7	-16°C Day 3	-16°C Day 7
10% TSB	VRBA	8.52	8.35	8.30	8.42	8.29	4.80	4.71
	T/V	8.63	8.61	8.56	8.56	8.54	7.55	7.26
TSB	VRBA	9.14	9.04	9.04	9.07	9.03	7.57	7.30
	T/V	9.22	9.04	9.53	9.25	9.48	8.62	8.42
NB	VRBA	8.23	8.37	8.29	8.36	8.15	5.43	4.37
	T/V	8.39	8.71	8.61	8.55	8.99	6.51	5.70
CM	VRBA	8.76	8.49	8.63	8.56	8.36	7.76	7.64
	T/V	8.72	8.87	9.07	8.83	8.49	8.32	8.14

TABLE 6. *Survival of* K. pneumoniae *ATCC 13883 Stored in Different Suspending Media at Various Temperatures*

Susp. Media	Plating Media	Initial No.	10°C Day 3	10°C Day 7	4°C Day 3	4°C Day 7	-16°C Day 3	-16°C Day 7
10% TSB	VRBA	8.11	7.93	7.95	7.48	7.33	3.95	4.79
	T/V	8.26	8.22	8.19	8.16	8.20	6.73	5.44
TSB	VRBA	8.92	8.75	8.86	8.86	8.76	6.82	6.85
	T/V	8.97	8.92	8.88	8.89	8.97	7.84	7.36
NB	VRBA	7.77	7.64	7.75	7.45	7.58	5.12	4.94
	T/V	8.09	8.12	8.12	8.09	8.03	5.81	5.83
CM	VRBA	8.67	8.31	7.83	8.39	8.42	7.11	6.53
	T/V	8.84	8.59	8.00	8.88	8.35	7.81	7.26

Key: TSB = Tryptic Soy broth
NB = Nutrient broth
CM = Cooked Meat medium
VRBA = Violet Red Bile agar
T/V = Tryptic Soy agar overlayered with Violet Red Bile agar (TSA/VRBA)

TABLE 7. *Survival of* K. pneumoniae *#2 Stored in Different Suspending Media at Various Temperatures*

Susp. Media	Plating Media	Initial No.	Survival of Cells (log cfu/ml) 10°C Day 3	10°C Day 7	4°C Day 3	4°C Day 7	-16°C Day 3	-16°C Day 7
10% TSB	VRBA	8.25	8.16	8.26	8.19	8.19	5.78	5.59
	T/V	8.53	8.53	8.75	8.30	8.38	7.38	7.34
TSB	VRBA	8.88	9.00	8.02	9.08	8.94	8.70	8.47
	T/V	9.28	9.28	9.38	9.27	9.29	9.01	9.06
NB	VRBA	8.26	8.19	8.23	8.21	8.12	4.88	5.10
	T/V	8.46	8.59	8.53	8.35	8.32	5.75	6.11
CM	VRBA	8.93	8.88	8.91	8.82	8.59	8.72	8.68
	T/V	9.21	9.04	9.20	8.97	8.76	9.06	8.99

TABLE 8. *Survival of* K. oxytoca *#1765 Stored in Different Suspending Media at Various Temperatures*

Susp. Media	Plating Media	Initial No.	10°C Day 3	10°C Day 7	4°C Day 3	4°C Day 7	-16°C Day 3	-16°C Day 7
10% TSB	VRBA	8.16	8.06	8.20	8.15	8.09	7.00	7.51
	T/V	8.37	8.46	9.16	8.20	8.16	8.01	7.82
TSB	VRBA	8.77	8.94	8.96	8.92	8.91	8.59	8.57
	T/V	9.11	9.09	9.66	8.96	9.09	9.01	8.94
NB	VRBA	8.29	7.98	8.10	8.05	7.96	5.49	4.92
	T/V	8.30	8.14	8.52	8.23	8.07	5.79	5.56
CM	VRBA	8.68	8.28	8.52	8.39	7.69	8.69	8.40
	T/V	8.84	8.53	8.81	8.43	7.86	8.92	8.61

Key: See bottom of Table 6, page 57.

Plating media also affected the apparent survival of the test cultures. Higher counts were obtained on the "repair" medium (TSA/VRBA) than on the selective medium (VRBA). This was especially noticeable at -16°C.

The data were analyzed to test for differences between the variables using the BMDP Repeated Measures analysis of variance (Program P2V, Dixon & Brown, 1981). The summary of results for the analysis is shown in Table 9. Highly significant interaction effects ($P<0.05$) were observed between storage temperature, time and broths. Significant individual effects of temperature, broth and time on the test cultures were expected, however, second and third level interactions were not expected. These highly significant interactions made the data difficult to interpret. As a result, the data were partitioned to test for effects of temperature, suspending medium and length of storage on the individual organisms in an attempt to determine what factors were contributing to the significant interactions that were observed. The summary of the analysis of variance for each organism is shown in Table 10.

These data indicate a marked difference in reaction to the storage conditions between the test cultures. *K. oxytoca* #1765 was not significantly affected by storage conditions and time of storage. *E. coli* 11775 was significantly affected ($P<0.05$) by storage temperature and time. There was a significant interaction effect between time and temperature of storage of *E. coli* 11775 which could be

TABLE 9. *Analysis of Variance for Effect of Storage Conditions on Survival of Test Cultures*

Source of Variation		Probability (P)
Organism	(O)	<0.0001
Storage broths	(L)	<0.0001
Temperature	(T)	<0.0001
OL		0.0538
OT		0.0046
LT		<0.0001
Days	(D)	<0.0001
DO		0.0003
DL		<0.0001
DT		<0.0001
DOL		0.1507
DOT		0.0011
DLT		<0.0001

TABLE 10. *Summary of Analyses of Variance for Effect of Storage Conditions and Time of Storage on Survival of Separate Test Cultures*

		Test Cultures[1]			
Source of Variation		1	2	3	4
		Probability (P)			
Storage broth	(L)	0.0742	0.0056	0.0519	0.0839
Temperature	(T)	0.0081	0.0009	0.0734	0.2309
Days	(D)	0.0005	<0.0001	0.0327	0.0565
DL		0.5732	0.3258	0.3991	0.3144
DT		0.0005	<0.0001	0.0250	0.1915

[1]Key: 1 = *E. coli* ATCC 11775, 2 = *K. pneumoniae* ATCC 13883, 3 = *K. pneumoniae* #2, 4 = *K. oxytoca* #1765

attributed to the organism surviving well on all media at 10 and 4°C, as well as on TSB and Cooked Meat medium at -16°C, but dying in 10%TSB and NB at -16°C. Similarly, time and temperature of storage had a significant interaction effect ($P<0.05$) for *K. pneumoniae* 13883 and #2. This effect could also be attributed to the poor survival of these organisms at -16°C in 10%TSB and NB. This is illustrated in Figure 1 for *K. pneumoniae* 13883 when grown on VRBA.

Death at -16°C, especially cultures stored in 10%TSB and NB, was much greater for the first three days than the last four days of storage. For most of the test cultures in 10%TSB and NB a 2 to 4 log decrease was frequently observed during the first 3 days of storage and minimal changes during the following 4 days of storage. Similarly, for *E. coli* 11775 and *K. pneumoniae* 13883 in TSB and Cooked Meat medium, there was a 1 to 2 log decrease after 3 days storage. Table 11 summarizes the descending order in which different broths supported survival of the test cultures. This was determined by comparing the log differences in cell numbers at each day of storage with the initial day 0 population for each broth. TSB was always among the better broths for survival of the cells, while 10%TSB was generally one of the poorer broths. However, the relative position of NB and Cooked Meat medium changed markedly with temperature of storage. This was particularly noticeable with Cooked Meat medium which was generally the poorest medium at 10 and 4°C, but the best at -16°C.

FIGURE 1. *Survival of* K. pneumoniae *ATCC 13883 Stored in Different Broths at 10, 4 and -16°C, and Enumerated on VRBA Medium*

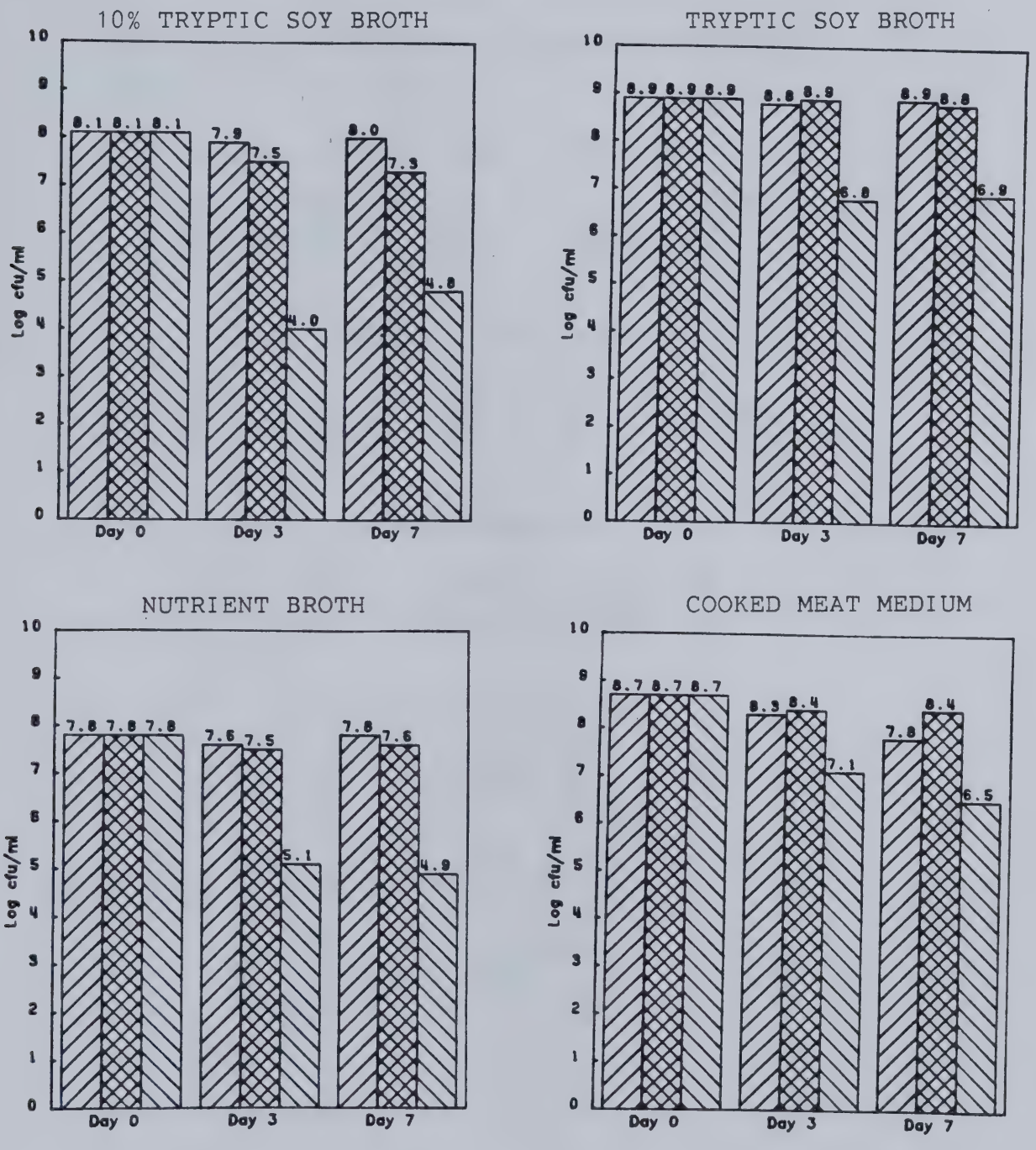


TABLE 11. *Summary of the Rank Order of the Protective Effect Attributed to Suspending Medium During Storage of Cells at Various Temperatures*

Storage Temperature	Rank Order of Survival in Broths[1]			
	1	2	3	4
	E. coli ATCC 11775			
10°C	NB	TSB	CM	10%TSB
4°C	NB	TSB	10%TSB	CM
-16°C	CM	TSB	10%TSB	NB
	K. pneumoniae ATCC 13883			
10°C	NB	TSB	10%TSB	CM
4°C	TSB	NB	CM	10%TSB
-16°C	CM	TSB	NB	10%TSB
	K. pneumoniae #2			
10°C	NB	TSB	10%TSB	CM
4°C	TSB	NB	10%TSB	CM
-16°C	CM	TSB	10%TSB	NB
	K. oxytoca #1765			
10°C	TSB	10%TSB	NB	CM
4°C	TSB	10%TSB	NB	CM
-16°C	CM	TSB	10%TSB	NB

[1] Rank order (highest to lowest) of survival of test cultures in broths:

TSB = Tryptic Soy broth
NB = Nutrient broth
CM = Cooked Meat medium

At the same time that the survival studies were done, isolates were picked from the plating media to detect phenotypic changes in the cells. Colonies were picked from TSA, TSA/VRBA and VRBA plates based on morphological differences such as colony size and the extent of bile precipitation on TSA/VRBA and VRBA plates; or, if no morphologically different colonies were observed, 3 colonies were picked at random from the plates. Colonies were also picked from TBA plates by replica plating, and based on the loss of indole producing characteristic on this medium. Of the four organisms used, *E. coli* ATCC 11775 and *K. pneumoniae* #2 are faecal coliforms, *K. pneumoniae* ATCC 13883 and *K. oxytoca* #1765 are non-faecal coliforms. The maximum temperature at which *K. pneumoniae* 13883 and *K. oxytoca* #1765 produced gas from lactose was 38° and 43°C, respectively.

Temporary and permanent variants were obtained for the four organisms from various combinations of the suspending broths and the plating media at the three storage temperatures. These variants were negative for gas production from lactose at 44.5°C or at the maximum temperature. *K. oxytoca* #1765 also produced variants that were negative for indole production from tryptophan at 43°C. A summary of the temporary and permanent variants detected for each organism under the storage conditions is shown in Table 12.

TABLE 12. *Percent of Temporary and Permanent Variants Detected when Test Cultures were Stored in Different Broths at Various Temperatures*

Organism	Percent of Variants Detected[1]			
	Lactose (gas)		Indole	
	Temporary	Permanent	Temporary	Permanent
E. coli ATCC 11775	4.7	-	-	-
K. pneumoniae ATCC 13883	33.0	1.7	NT	NT
K. pneumoniae #2	9.5	-	NT	NT
K. oxytoca #1765	20.0	20.0	70.0	45.5

[1]Variants were detected at the elevated temperature of 44.5°C or at the maximum temperature for the organism.

Key: Lactose (gas) = lactose fermented with gas production

- = no variants detected

NT = not tested for that characteristic

A total of 214 colonies of *E. coli* ATCC 11775 was selected. Of the 214 colonies, 10 (4.7 percent) temporary variants were detected on the 3rd day of storage. The variants were obtained at all storage temperatures, from a variety of the suspending broths and from both TSA and TSA/VRBA plates. No variants were detected on the 7th day of storage. After subculturing in NB for 3 consecutive days, all variants regained their original biochemical characteristics.

A total of 181 colonies of *K. pneumoniae* ATCC 13883 was selected. Variants were detected among isolates from both the 3rd and 7th days of storage. Of the 181 colonies, 60 (33 percent) temporary variants were detected at all of the storage temperatures used. They were isolated from all of the suspending broths and from TSA, VRBA and TSA/VRBA plating media. Four of the variants gave questionable reactions in TSI, producing an orange colour throughout the medium, instead of the yellow colour that indicates acid production from the fermentable carbohydrates. After subculturing in NB for 3 consecutive days, only 3 (1.7 percent) permanent variants were detected. These 3 variants were detected on the 3rd day of storage and were negative for gas from lactose at 38°C. One variant was obtained from the culture stored in Cooked Meat medium at 4°C and plated on VRBA; the other 2 were obtained from cultures stored in 10%TSB and Cooked Meat medium at -16°C, and plated on TSA/VRBA and VRBA, respectively.

A total of 147 colonies of *K. pneumoniae* #2 was selected. Of the 147 colonies, 2 (1.4 percent) variants were detected from cultures stored in 10%TSB and NB at -16°C on the 3rd day of storage, and plated onto TSA/VRBA. On the 7th day of storage, 12 (8.2 percent) variants were detected from cultures stored in each of the liquid media and plated onto TSA/VRBA. All the variants were negative for gas from lactose at 44.5°C, except one, which gave an alkaline reaction on TSI agar slants. After subculturing in NB for 3 consecutive days, all variants regained their original biochemical characteristics.

A total of 156 colonies of *K. oxytoca* #1765 was selected. Temporary and permanent variants were detected on both the 3rd and 7th days of storage. Of the 156 colonies, 109 temporary variants (70 percent) were detected that were negative for the production of indole from tryptophan at 43°C; 31 (20 percent) were detected that were negative for the production of gas from lactose fermentation at 43°C. These variants were obtained from various combinations of the suspending broths and the plating media at the three storage temperatures. After subculturing in NB for three consecutive days, 71 variants (45.5 percent) remained negative for the production of indole from tryptophan and 31 variants (20 percent) remained negative for gas production from lactose fermentation at 43°C. Among the temporary and permant variants detected, some were negative for both characteristics.

Survival of Test Cultures Stored in Ground Beef

In the previous experiment, the test cultures grew slowly when stored at 10°C, and no marked differences were observed between storage at 10 and 4°C. In contrast, marked differences in survival of the cells were observed when the organisms were stored at -16°C. In this experiment, the test cultures were stored in ground beef. Storage temperatures of 4 and -16°C were retained, and -40°C was included. Storage at -40°C was selected to simulate commercial conditions for frozen storage of foods. In addition, a reference strain of *K. oxytoca*, ATCC 13182 was included, increasing the number of test cultures to five. Survival of the organisms was measured by plating onto TSA, VRBA, TSA/VRBA and MCIC after 3 and 7 days storage at the selected storage temperatures.

In the early stages of this study, it was observed that at 4°C, by the 3rd day of storage, there was usually a 1 log decrease in the population of the test cultures. As a result, survival of cells on each day of storage at 4°C was tested up to day 3. This experiment includes observations for samples stored at 4°C on the initial day and on days 1, 2, 3 and 7 of storage. At -16 and -40°C, observations were made initially and on days 3 and 7 of storage. The log data for survival of each organism, under each condition of storage, are shown in Tables 13 and 14. Storage at 4°C in meat had a marked effect on survival of the organisms compared to storage at -16 and -40°C. The *Klebsiella* cultures stored at 4°C decreased by as much as 1 log cycle

TABLE 13. *Survival of the Test Cultures Stored in Ground Beef at 4°C for 7 Days*

Organism	Plating Media	Initial Count	Survival of Cells' (log cfu/g) Day 1	Day 2	Day 3	Day 7
E. coli	VRBA	7.32	7.21	6.99	6.88	6.52
ATCC 11775	T/V	7.40	7.33	7.15	7.05	6.67
K. pneumoniae	VRBA	7.14	6.99	6.81	6.51	5.83
ATCC 13883	T/V	7.24	7.14	7.04	6.84	6.18
K. pneumoniae	VRBA	6.98	6.93	6.77	6.69	5.88
#2	T/V	7.04	7.06	7.05	6.69	6.80
K. oxytoca	VRBA	6.98	6.92	6.63	6.35	5.78
ATCC 13182	T/V	7.00	6.95	6.71	6.58	6.09
K. oxytoca	VRBA	6.91	6.87	6.61	6.40	5.96
#1765	T/V	7.05	7.02	6.80	6.58	6.15

TABLE 14. *Survival of Test Cultures Stored in Ground Beef at -16 and -40°C for 7 Days*

Organism	Plating Media	Initial Count	-16°C Day 3	-16°C Day 7	-40°C Day 3	-40°C Day 7
E. coli	VRBA	7.31	6.91	6.77	7.02	7.05
ATCC 11775	T/V	7.64	7.34	7.20	7.37	7.29
K. pneumoniae	VRBA	7.04	6.86	6.44	6.71	6.68
ATCC 13883	T/V	7.19	7.06	7.02	7.04	7.06
K. oxytoca	VRBA	6.91	6.93	6.87	6.80	6.83
#1765	T/V	7.05	7.03	6.97	7.01	6.84

Key: VRBA = Violet Red Bile agar
T/V = Tryptic Soy agar overlayered with Violet Red Bile agar (TSA/VRBA)

'The concentration of the test organisms used to inoculate the ground beef was sufficiently high to outgrow the natural meat flora. As a result, virtually pure cultures were obtained. The numbers of colony forming units recorded in the Tables, therefore, represent the test cultures.

by the 7th day of storage. For *E. coli*, the decrease was less than 1 log cycle by day 7. In contrast, less than 1 log cycle of the population died when stored at -16 and -40°C. Of the organisms tested at -16 and -40°C, *E. coli* ATCC 11775 survived better at -40 than at -16°C, while *K. pneumoniae* ATCC 13883 and meat strain *K. oxytoca* #1765 survived better at -16°C.

The data were statistically analysed to test for significant differences between the variables. A Repeated Measures analysis of variance of the data for storage of the 5 organisms at 4°C gave significantly high interaction effects ($P<0.05$) between storage broths, temperature and time, similar to the previous experiment. As a result, the data were more closely examined in an attempt to determine the cause of these effects by plotting the log of the mean cell numbers against storage time. It was observed that the rate of decrease by the 3rd day of storage was similar for all the cultures. However, by the 7th day, there was a greater decrease with the *Klebsiella* cultures (just over 1 log cycle) than with *E. coli* 11775.

The data were partitioned so that statistical tests could be done on the 3 individual organisms stored at 4, -16 and -40°C (Table 15). Interaction effects between the time and temperature of storage for *K. pneumoniae* ATCC 13883 and *K. oxytoca* #1765 were observed. In addition, storage time had a significant effect ($P<0.05$) on all three organisms, while storage temperature was significant only for *K.*

TABLE 15. *Summary of Analyses of Variance for Effect of Time and Temperature of Storage on Survival of Test Cultures*

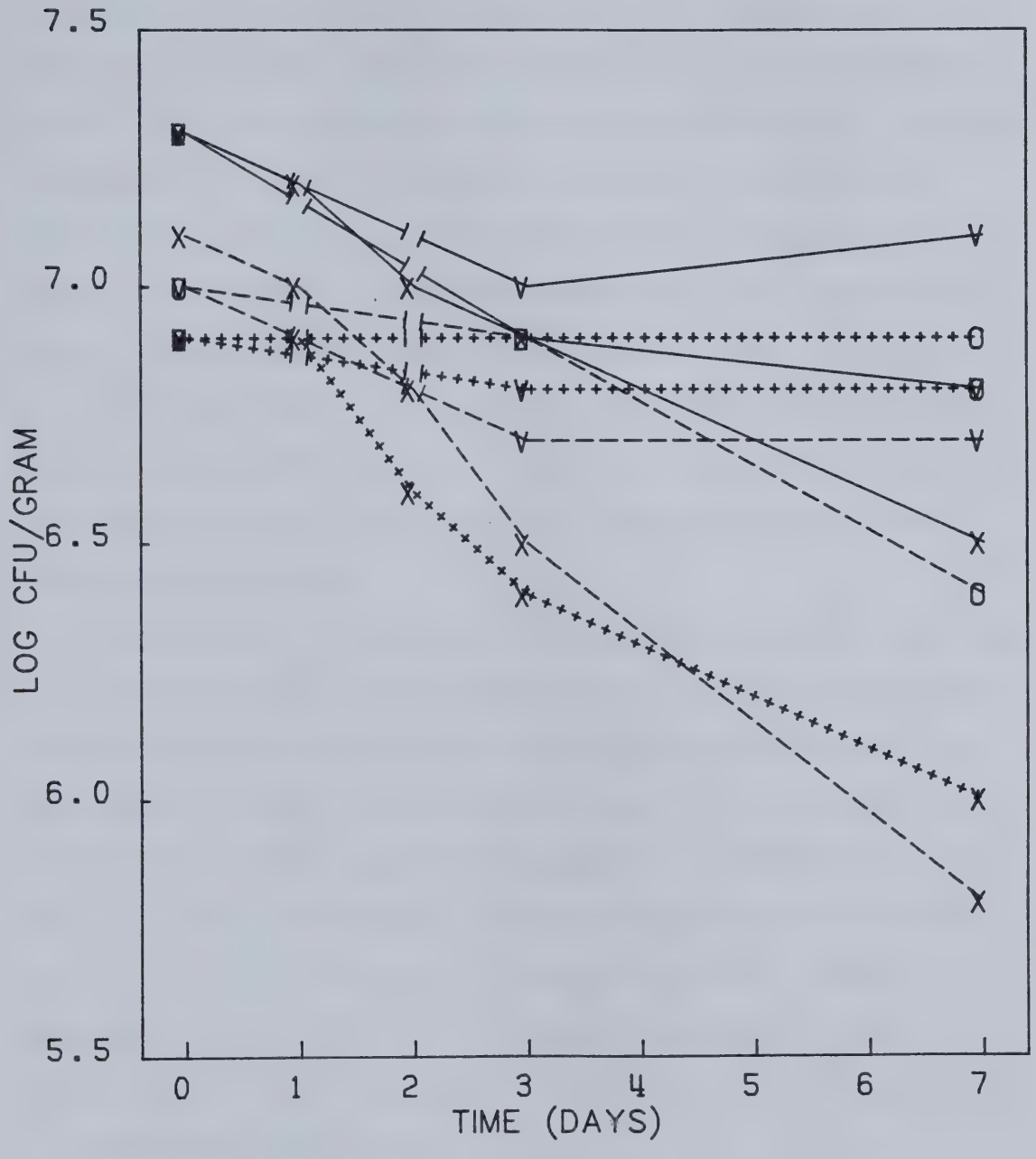
	E. coli ATCC 11775	*K. pneumoniae* ATCC 13883	*K. oxytoca* #1765
	Probability (P)		
Temperature	0.0720	0.0859	0.0025
Replicate (R)	0.2737	0.3513	0.4676
Days (D)	0.0001	0.0002	0.0015
DT	0.0877	0.0267	0.0023
DR	0.6706	0.8344	0.1160

oxytoca #1765. The interaction effects between time and temperature of storage were more closely examined by plotting the data (Figure 2). These effects could be attributed to the poorer survival of the *Klebsiella* organisms at 4°C compared to *E. coli*. At 4°C by the 2nd day of storage, the rate of decrease was similar for all 3 organisms. However, from the 2nd to the 7th day of storage, there was a marked decrease in the survival of *K. pneumoniae* ATCC 13883 and *K. oxytoca* #1765. This was not the case at -16 and -40°C.

The daily recording of the pH values of the meat showed no appreciable difference from day to day. The initial pH of the ground beef was between 5.6 and 5.7. Between day 1 and day 3, the pH of the inoculated ground beef ranged between 5.5 and 5.7. The lowest pH readings were observed on the 3rd day of storage, being 0.05 to 0.20 pH units lower than the initial reading. The highest pH was generally recorded on day 7 in the meats stored at 4°C, and it was usually greater than or equal to 6.0.

Isolates were also picked to determine phenotypic changes in the cells under these conditions of storage. Only two of the test organisms were studied, *E. coli* ATCC 11775 and *K. pneumoniae* ATCC 13883. These studies were limited by the fact that the test organisms were no longer in pure culture, and in the case of TBA, the medium was overgrown with other organisms from the meat flora by the 7th day of storage at 4°C.

FIGURE 2. *Survival of* E. coli *ATCC 11775,* K. pneumoniae *ATCC 13883 and* K. oxytoca *#1765 in Ground Beef Stored at 4, -16 and -40°C, and Enumerated on VRBA Medium*



Key:

—— *E. coli* 11775 X = storage at 4°C.

----- *K. pneumoniae* 13883 O = storage at -16°C.

••••••• *K. oxytoca* 1765 V = storage at -40°C.

No permanent variants were obtained for *E. coli* 11775. Unlike the isolates of the broth study, all of the variants detected were normal for gas production from lactose, but they were negative for indole production from tryptophan at 44.5°C. Temporary variants were detected at 3 and 7 days of storage. Of the the 55 colonies selected to test for biochemical variants, 42 (76 percent) were found to be temporary variants for indole production from tryptophan. Of these, 9 variants were detected at 4°C, 17 at -16°C and 16 at -40°C. They were isolated from TSA, VRBA and TSA/VRBA plates. After subculturing in NB for 3 consecutive days, all variants regained their ability to produce indole from tryptophan at 44.5°C.

A total of 73 colonies of *K. pneumoniae* ATCC 13883 was selected. Variants for gas production from lactose at the maximum temperature of 38°C, were obtained on the 3rd and 7th days of storage. During this time, 14 (19 percent) of the isolates were found to be permanent variants. Two of these were obtained from cultures stored at 4°C and 6 each at -16 and -40°C. They were isolated from TSA, VRBA and TSA/VRBA plates. All of the variants remained negative for gas production from lactose at 38°C, after subculturing for 3 consecutive days in NB.

Survival of the Test Cultures Stored in Broths and Ground Beef Adjusted to Different pH Levels

From the previous experiments, it appeared that survival of the test cultures was challenged by storage in certain broths, and by storage at 4°C in ground beef. This experiment was designed to determine factors that might influence survival of the test cultures under these conditions, in particular pH of the storage medium and presence of the indigenous meat microflora.

Tubes of NB and TSB were adjusted to pH 5.5, 6.0, and 6.5 and inoculated with the test cultures. They were stored at 4°C for 3 days. The results are shown in Tables 16 to 20. Survival of the test cultures was generally similar at all pH levels. Plating medium influenced the number of survivors observed in the broths. In most cases, the "repair" medium (TSA/VRBA) supported the growth of more colonies than the selective medium (VRBA or MCIC). However, differences were not usually greater than a factor of two, and trends for survival on both media were generally the same. Nonetheless, the differences indicated that injury had occurred. During the 3 days of storage in broths, at the 3 pH levels, a slight decrease in count was recorded for all test cultures.

The data were analysed using the BMDP Repeated Measures design to test for statistically significant differences between the variables. The results for the analysis of variance are summarized in Table 21. There were highly significant levels ($P<0.05$) of interaction between the

TABLE 16. *Survival of* E. coli *ATCC 11775 Stored in NB and TSB Adjusted to Different pH Levels, at 4°C for 3 Days*

		Survival of Cells (log cfu/ml)							
	Suspending media:	NB				TSB			
	Plating	Storage time				Storage time			
pH	Media	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
5.5	VRBA	8.30	8.17	8.05	8.05	8.36	8.28	8.12	8.13
	T/V	8.35	8.42	8.36	8.36	8.40	8.45	8.29	8.26
6.0	VRBA	8.33	8.16	8.55	7.94	8.34	8.22	7.98	7.95
	T/V	8.24	8.47	8.34	8.34	8.48	8.41	8.24	8.21
6.5	VRBA	8.35	8.25	8.02	7.86	8.33	8.24	7.95	7.70
	T/V	8.60	8.44	8.35	8.31	8.42	8.44	8.24	8.09

TABLE 17. *Survival of* K. pneumoniae *ATCC 13883 Stored in NB and TSB Adjusted to Different pH Levels, at 4°C for 3 Days*

	Suspending media:	NB				TSB			
	Plating	Storage time				Storage time			
pH	Media	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
5.5	MCIC	7.88	7.55	7.59	7.23	7.95	7.56	7.68	7.52
	T/V	8.29	8.14	8.10	7.99	8.19	8.18	8.14	8.16
6.0	MCIC	7.63	7.43	7.54	7.11	7.97	7.45	7.31	7.18
	T/V	8.24	8.10	7.98	7.73	8.30	8.11	8.13	8.01
6.5	MCIC	7.32	7.58	7.10	6.79	8.04	7.48	7.46	6.91
	T/V	8.38	8.13	7.74	7.41	8.29	8.15	8.03	7.71

Key: VRBA = Violet Red Bile agar
MCIC = MacConkey Inositol Carbenicillin agar
T/V = Tryptic Soy agar overlayered with Violet Red Bile agar (TSA/VRBA)

TABLE 18. *Survival of* K. pneumoniae *#2 Stored in NB and TSB Adjusted to Different pH Levels, at 4°C for 3 Days*

		Survival of Cells (log cfu/ml)							
	Suspending media:	NB				TSB			
	Plating	Storage time				Storage time			
pH	Media	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
5.5	MCIC	8.32	8.21	8.12	8.05	8.40	8.37	8.31	8.27
	T/V	8.40	8.34	8.28	8.15	8.42	8.51	8.39	8.36
6.0	MCIC	8.34	8.23	8.03	8.02	8.35	8.41	8.28	8.26
	T/V	8.43	8.35	8.14	8.16	8.43	8.52	8.37	8.33
6.5	MCIC	8.34	8.20	8.06	7.96	8.37	8.45	8.33	8.25
	T/V	8.39	8.29	8.20	8.14	8.44	8.26	8.34	8.38

TABLE 19. *Survival of* K. oxytoca *ATCC 13182 Stored in NB and TSB Adjusted to Different pH Levels, at 4°C for 3 Days*

	Suspending media:	NB				TSB			
	Plating	Storage time				Storage time			
pH	Media	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
5.5	MCIC	8.20	8.11	8.00	7.98	8.19	8.14	8.07	8.11
	T/V	8.32	8.32	8.30	8.20	8.35	8.32	8.24	8.24
6.0	MCIC	8.28	8.07	7.90	7.89	8.20	8.18	8.16	8.02
	T/V	8.33	8.35	8.18	8.14	8.30	8.38	8.23	8.16
6.5	MCIC	8.24	8.08	7.95	7.82	8.18	8.18	8.12	8.02
	T/V	8.38	8.35	8.23	8.12	8.37	8.38	8.20	8.19

Key: See bottom of Table 17, page 76.

TABLE 20. *Survival of* K. oxytoca *#1765 Stored in NB and TSB Adjusted to Different pH Levels, at 4°C for 3 Days*

		Survival of Cells (log cfu/ml)							
	Suspending media:	NB				TSB			
	Plating	Storage time				Storage time			
pH	Media	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
5.5	MCIC	8.04	8.10	7.91	7.66	8.00	8.07	8.01	7.99
	T/V	8.18	8.08	7.99	7.81	8.12	8.11	8.11	8.06
6.0	MCIC	7.96	7.86	7.71	7.56	8.04	8.06	8.00	7.87
	T/V	8.12	8.04	7.94	7.77	8.14	8.11	8.06	8.01
6.5	MCIC	7.96	7.95	7.73	7.46	7.98	8.00	7.91	7.72
	T/V	8.11	7.95	7.84	7.77	8.12	8.11	8.01	7.90

Key: See bottom of Table 17, page 76.

TABLE 21. *Analyses of Variance for Effect of pH and Broth on survival of Test Cultures Stored at 4°C for 3 Days*

Source of Variation	Probability (P) Selective Medium (VRBA or MCIC)	Repair Medium (TSA/VRBA)
Organism (O)	<0.0001	<0.0001
Treatment (T)	<0.0001	<0.0001
Replicate (R)	<0.0001	0.0007
OT	0.0011	0.0007
OR	<0.0001	0.0031
TR	0.2074	0.9982
Days (D)	<0.0001	<0.0001
DO	<0.0001	<0.0001
DT	<0.0001	<0.0001
DR	0.1823	0.0158
DOT	0.0001	<0.0001
DOR	0.0044	0.0015
DTR	0.2552	0.0687

variables; as a result, the data were partitioned into analyses for each of the test organisms, and the results are shown in Table 22. Treatments, consisting of the two broth media, each adjusted to 3 pH levels, had a significant effect ($P<0.05$) on the survival of all organisms except *E. coli* ATCC 11775, and *K. oxytoca* ATCC 13182 when it was plated on the "repair" medium. Storage time (days) had a significant effect on the survival of all test organisms. Some significant interaction effects were recorded, however they were not further analysed because the reductions in the counts during the storage time were less than 1 log cycle, which makes them of little practical significance.

The daily recording of the pH values showed changes no greater than 0.02 pH units.

Before inoculation with the test cultures, the ground beef was subjected to different treatments. A sample of ground beef was divided into 5 portions; three were adjusted to pH 5.5, 6.0, and 6.5; the other two were stored at 10°C for 24 h, one under anaerobic conditions (vacuum packaged), the other under aerobic conditions. This was done to allow the growth of the natural meat microflora. All samples were then inoculated with the test cultures and stored at 4°C for 3 days. The mean pH of the "vacuum packaged" beef was 5.7 (range 5.6 - 5.7), and of the "aerobic" beef was 5.8 (range 5.7 - 5.8). The data for survival of the test cultures in meat under these conditions are shown in Tables 23 to 27.

TABLE 22. *Summary of the Analyses of Variance for pH and Broth Effect on Survival of Separate Test Cultures*

Source of Variation		Test Cultures				
(VRBA or MCIC)		1	2	3	4	5
		Probability (P)				
Treatment	(T)	0.1832	0.0229	0.0008	0.0130	0.0005
Replicate	(R)	0.5555	0.0004	0.1228	0.0031	0.0183
Days	(D)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
DT		0.0126	0.0016	0.3929	0.0709	0.0678
DR		0.7547	0.0118	0.3839	0.0571	0.1374
(TSA/VRBA)						
Treatment	(T)	0.3753	0.0001	0.0104	0.7447	0.0018
Replicate	(R)	0.2067	0.0005	0.0261	0.0086	0.0468
Days	(D)	0.0001	<0.0001	0.0003	<0.0001	<0.0001
DT		0.1048	<0.0001	0.3584	0.0428	0.0171
DR		0.0505	0.0693	0.0851	0.1592	0.0846

Key: 1 = *E. coli* ATCC 11775; 2 = *K. pneumoniae* ATCC 13883;

3 = *K. pneumoniae* #2; 4 = *K. oxytoca* ATCC 13182;

5 = *K. oxytoca* #1765

TABLE 23. *Survival of* E. coli *ATCC 11775 Stored in Ground Beef Adjusted to Different pH Levels, at 4°C for 3 Days*

pH	Plating Media	Survival of Cells (log cfu/g)[1] Storage Time Day 0	Day 1	Day 2	Day 3
5.5	VRBA	7.15	6.96	6.87	6.54
	TSA/VRBA	7.16	7.19	7.05	6.95
6.0	VRBA	7.16	7.00	6.56	6.57
	TSA/VRBA	7.19	7.19	6.99	6.91
6.5	VRBA	7.19	7.02	6.88	6.77
	TSA/VRBA	7.34	7.21	7.14	7.10
Vacuum Packaged	VRBA	7.11	6.67	6.60	6.33
	TSA/VRBA	7.61	7.18	6.99	6.85
Aerobic	VRBA	7.07	7.10	7.09	6.78
	TSA/VRBA	7.26	7.15	7.12	7.03

TABLE 24. *Survival of* K. pneumoniae *ATCC 13883 Stored in Ground Beef Adjusted to Different pH Levels, at 4°C for 3 Days*

pH	Plating Media	Storage Time Day 0	Day 1	Day 2	Day 3
5.5	MCIC	6.70	6.16	5.81	5.52
	TSA/VRBA	6.92	6.63	6.22	5.96
6.0	MCIC	6.76	6.14	5.93	5.57
	TSA/VRBA	6.92	6.60	6.28	6.03
6.5	MCIC	6.75	6.15	5.93	5.50
	TSA/VRBA	6.93	6.61	6.33	6.11
Vacuum Packaged	MCIC	6.62	6.33	6.07	5.96
	TSA/VRBA	6.80	6.86	6.52	6.45
Aerobic	MCIC	6.59	6.33	6.08	5.93
	TSA/VRBA	6.92	6.76	6.57	6.46

[1]See footnote at bottom of Table 14, page 69.

Key: VRBA = Violet Red Bile agar
MCIC = MacConkey Inositol Carbenicillin agar
T/V = Tryptic Soy agar overlayered with Violet Red Bile agar (TSA/VRBA)

TABLE 25. *Survival of* K. pneumoniae *#2 Stored in Ground Beef Adjusted to Different pH Levels, at 4°C for 3 Days*

pH	Plating Media	Survival of Cells (log cfu/g)[1] Storage Time Day 0	Day 1	Day 2	Day 3
5.5	MCIC	7.10	6.66	6.74	6.63
	TSA/VRBA	7.15	7.04	6.91	6.82
6.0	MCIC	7.25	6.68	6.83	6.77
	TSA/VRBA	7.21	7.07	6.96	6.99
6.5	MCIC	7.20	7.11	7.02	6.97
	TSA/VRBA	7.25	7.16	7.05	7.12
Vacuum Packaged	MCIC	6.70	6.91	6.65	6.58
	TSA/VRBA	7.51	7.03	6.86	6.74
Aerobic	MCIC	7.02	7.03	6.73	6.55
	TSA/VRBA	7.15	7.05	6.92	6.80

TABLE 26. *Survival of* K. oxytoca *ATCC 13182 Stored in Ground Beef Adjusted to Different pH Levels, at 4°C for 3 Days*

pH	Plating Media	Storage Time Day 0	Day 1	Day 2	Day 3
5.5	MCIC	6.85	6.84	6.85	6.76
	TSA/VRBA	6.82	7.02	6.95	6.94
6.0	MCIC	6.89	6.88	6.89	6.90
	TSA/VRBA	6.97	7.08	7.07	7.09
6.5	MCIC	6.95	6.92	6.99	6.70
	TSA/VRBA	7.01	7.17	7.16	7.22
Vacuum Packaged	MCIC	6.82	6.81	6.77	6.76
	TSA/VRBA	7.07	6.97	7.09	6.89
Aerobic	MCIC	6.70	6.87	6.80	6.85
	TSA/VRBA	7.14	7.11	7.12	6.95

[1]See footnote at bottom of Table 14, page 69.

Key: See bottom of Table 24, page 82.

TABLE 27. *Survival of* K. oxytoca *#1765 Stored in Ground Beef Adjusted to Different pH Levels, at 4°C for 3 Days*

pH	Plating Media	Survival of Cells (log cfu/g)[1] Storage Time Day 0	Day 1	Day 2	Day 3
5.5	MCIC	6.54	6.77	6.55	6.40
	TSA/VRBA	6.87	6.72	6.67	6.61
6.0	MCIC	6.69	6.75	6.66	6.52
	TSA/VRBA	6.85	6.91	6.86	6.74
6.5	MCIC	6.66	6.93	6.83	6.60
	TSA/VRBA	6.86	6.92	6.98	6.81
Vacuum Packaged	MCIC	6.49	6.34	6.25	6.09
	TSA/VRBA	6.68	6.59	6.36	6.27
Aerobic	MCIC	6.55	6.40	6.08	6.09
	TSA/VRBA	6.75	6.63	6.29	6.23

[1]See footnote at bottom of Table 14, page 69.

Key: See bottom of Table 24, page 82.

From the data, it was observed that death of *E. coli* 11775 and *K. pneumoniae* 13883 occurred during storage. The extent of death of *K. pneumoniae* meat strain #2 was less marked, but both of the *K. oxytoca* strains survived well. No marked differences in survival were observed as a result of the different pH levels. The pretreatments of vacuum packaging and aerobic storage had variable effects on survival of the test organisms. In general, however, the test cultures survived slightly better in the "aerobic" meats than in the "vacuum packaged" meats. Within the 3 days of storage, loss of viability was minimal.

The data were statistically analysed using the BMDP Repeated Measures design to test for significant effect of the variables on survival of the organisms. From the summary of the analysis of variance shown in Table 28, it may be seen that high levels of interaction ($P<0.05$) were again observed for data from the repair medium, but not from the selective media. The data were partitioned to measure the effects of the storage treatments for each of the test cultures. The summary of these analyses is shown in Table 29. In some cases, treatments gave a significant effect on survival of the organisms, storage time was always significant, except for *K. oxytoca* ATCC 13182 grown on the selective medium. There were some significant interaction effects, notably with *K. pneumoniae* ATCC 13883 and *K. oxytoca* #1765. In the case of *K. pneumoniae* 13883, these interaction effects could be attributed to better survival

TABLE 28. *Analysis of Variance for Effect of pH on Survival of Test Cultures Stored in Ground Beef at 4°C for 3 Days*

Source of Variation	Probability (P)	
	Selective Media	Repair Media
Organism (O)	<0.0001	<0.0001
Treatment (T)	0.0019	0.2317
Replicate (R)	0.2022	0.8936
OT	0.0054	0.2665
OR	0.0259	0.2849
TR	0.2784	0.3402
Days (D)	<0.0001	<0.0001
DO	<0.0001	<0.0001
DT	0.6681	0.0020
DR	0.0325	0.0018
DOT	0.0840	<0.0001
DOR	0.4807	0.0002
DTR	0.8862	0.0014

TABLE 29. *Summary of Analyses of Variance for Effect of Storage of Test Cultures in Ground Beef*

Source of Variation		Test Cultures 1	2	3	4	5
(VRBA or MCIC)		Probability (P)				
Treatment	(T)	0.3417	0.1118	0.0585	0.2756	0.0088
Replicate	(R)	0.7696	0.0529	0.4169	0.5389	0.0187
Days	(D)	<0.0001	<0.0001	0.0037	0.4321	<0.0001
DT		0.4547	0.0237	0.4031	0.5669	0.0378
DR		0.8016	0.0838	0.6958	0.0491	0.0061
(TSA/VRBA)						
Treatment	(T)	0.9909	0.0189	0.0850	0.0022	0.0042
Replicate	(R)	0.5214	0.1285	0.0706	0.0306	0.2570
Days	(D)	<0.0001	<0.0001	<0.0001	0.0404	<0.0001
DT		0.2030	0.0002	0.1776	0.0072	0.0175
DR		0.8722	0.0025	0.0292	0.0463	0.0094

Key: 1 = *E. coli* ATCC 11775; 2 = *K. pneumoniae* ATCC 13883;
3 = *K. pneumoniae* #2; 4 = *K. oxytoca* ATCC 13182;
5 = *K. oxytoca* #1765.

of the organism in meats in which the natural microflora had been allowed to develop, as opposed to those in which the pH had been adjusted. The reduction in the test cultures during storage had no practical significance for the *K. oxytoca* strains, but for *E. coli* and *K. pneumoniae* the extent of reduction could be of practical significance for detection of these bacteria in meats.

The daily recording of the pH values of the meat showed no appreciable difference from day to day. The pH fluctuated by ±0.2 pH units.

VI. DISCUSSION AND CONCLUSIONS

It was observed by Stiles and Ng (1981) that *K. pneumoniae* (*sensu lato*) was isolated as one of the dominant Enterobacteriaceae in meats at packing plants, but that it was less frequently found in meats at retail level. Based on this observation, it was suggested that *K. pneumoniae* (*sensu lato*) might not survive well in meats, and if that is the case, there is the possibility that it might be used as a more meaningful indicator organism for sanitation of meats than *E. coli*.

This study was undertaken to examine the survival of *K. pneumoniae* and *K. oxytoca* in liquid bacteriological media and in ground beef, to determine possible reasons for their low recovery from retail meats. There were two possibilities for the decrease in *K. pneumoniae*: either that storage of meats at low temperatures results in a significant loss of viability, or that the failure to detect *K. pneumoniae* (*sensu lato*) results from phenotypic changes, brought about by the storage conditions, such as refrigeration, freezing and thawing, and prolonged storage at low temperatures.

Several factors are known to affect the survival of bacterial cells during low temperature storage. These include the temperature at which the cells are held, the nature of the suspending medium and the length of storage. The first part of this study dealt with the survival of *K. pneumoniae* and *K. oxytoca* stored at various temperatures in broths of differing nutrient composition. Storage

temperatures that simulate conditions found in food handling were selected for use in the study.

Storage at 10 and 4°C was selected to represent poor and acceptable refrigeration, respectively. Health departments recommend 4°C as the storage temperature for the display of meats and other foods. Frozen storage at -16±2°, and -40°C was selected to represent unstable and stable freezing temperatures for foods.

Experiments with the test cultures in suspending media were done as a pilot study to determine whether survival of the cells or phenotypic change was most likely to account for the apparent disappearance of *K. pneumoniae* stored in meats. From this study it appeared that *K. pneumoniae* (*sensu lato*) dies rapidly under certain conditions, especially at -16°C. There was some change in phenotype for the characteristics tested, including ability to produce gas from lactose and indole from tryptophan at elevated or maximum incubation temperatures. These changes occurred as a result of all treatments, for all of the test cultures. However, in contrast to the findings of Bueschkens (1982), most of the variants were temporary rather than permanent in nature. Variants generally recovered their original phenotype after three subcultures suggesting injury or temporary inhibition of the enzyme system rather than a genetic change.

The effect of the storage treatments on survival of the test cultures was not uniform. Not only the specific test

organism, but also suspending medium and temperature at which they were stored had a varying effect on survival of the cells. However, death as great as 4 log cycles (99.99 percent) of cells as a result of some treatments indicated that death of *K. pneumoniae* was a significant possibility in the disappearance of these organisms during cold storage, especially during frozen storage at -16°C.

The suspending media were all common bacteriological broths except 10%TSB, which had been used by Bueschkens (1982) to study the effect of cryoprotective agents. In this study, the four broths were selected because of their different ingredients. TSB is a general purpose growth medium for fastidious organisms and contains peptones and carbohydrates that should be cryoprotective (Ray & Speck, 1973). In contrast, nutrient broth contains fewer ingredients and protective agents. Cooked Meat medium was incorporated in the experiment for its relationship to meat and the possibility that it might indicate a difference between the bacteriological media and meat.

Marked differences between media were apparent, and these differences were influenced by temperature of storage. Cooked Meat medium supported the lowest survival of cells at 10 and 4°C, but the greatest survival at -16°C. This was observed for all 4 test cultures (see Table 11). TSB generally supported the survival of the test organisms, and it was always better than 10%TSB. Suspending medium obviously had a marked effect on survival of these test

cultures during storage, even if the effect on survival of the different test organisms varied.

Because of the similarities in the survival of the test cultures at 10 and 4°C, no further studies were done at 10°C. However, the marked death of organisms stored at -16°C, warranted further study of viability during frozen storage. As a result, the more stable freezing temperature of -40°C was also included in the ground beef experiment. In addition, another reference organism, *K. oxytoca* ATCC strain 13182, was included because the meat strain of *K. oxytoca* was not significantly affected by the storage treatments.

In the meat studies, the test cultures were inoculated into ground beef at counts of 10^7 to 10^8 cfu/g. This meant that there would be little or no interference from the residual Enterobacteriaceae, or *E. coli* and *K. pneumoniae* organisms. In contrast to the broth studies, survival was poor at 4°C and good at both freezer temperatures (-16 and -40°C). Again marked differences were observed between the test organisms. Each of the *Klebsiella* test cultures decreased in viability when stored in the suspending broths at 4°C and at a slightly faster rate than was observed for the *E. coli* reference strain. The death of the cells was generally 1 log cycle (90 percent) by the 7th day of storage. Death of this number of cells could account for the recorded disappearance of *K. pneumoniae* between packing plant and retail levels of meat handling (Stiles & Ng, 1981), and further supports the possibility of using *K.*

pneumoniae as an indicator organism for unsanitary handling of meats.

Changes in ability of *E. coli* to produce indole from tryptophan and *K. pneumoniae* to produce gas from lactose at elevated and maximum incubation temperatures occurred at all storage temperatures. Indole negative variants regained their original biochemical characteristics after three subcultures, whereas some lactose variants were permanent changes. Although there were no permanent variants that were negative for the production of gas from lactose fermentation or indole from tryptophan at the elevated temperature of 44.5°C, the detection of temporary variants is significant in the food industry. It means that in a routine examination of food for faecal coliforms, using the faecal coliform test criteria, some of these organisms that have temporarily lost the ability to ferment lactose aerogenically at 44.5°C will not be detected. As a result, a potentially hazardous food may be accepted.

The fact that *Klebsiella* variants were detected that were negative for gas production from lactose at their maximum temperature could mean that these organisms were previously aerogenic at 44.5°C. Constant exposure to an extraenteral environment could have resulted in a gradual decrease in the maximum temperature at which they produce gas from lactose. This speculation has significance for the future acceptance of *Klebsiella* as a faecal indicator, but it indicates that both viability of the cells at 4°C and

phenotypic change could account for the disappearance of *K. pneumoniae* from meats. From the data in this study, however, it appears that loss of viability might be a more significant factor than phenotypic changes.

A meat system, such as fresh ground beef, is dynamic and the environment in the meat changes as a result of the growth of the indigenous meat microflora. Not only the number of psychrotrophic bacteria that grow during refrigeration (4°C) to 7 days, but also their metabolic products, could affect the survival of the test cultures. It therefore seemed appropriate to determine the effect of pH and microbial load of the ground beef on the survival of the test cultures at 4°C. As a result the final experiment was designed to determine the effect of pH, the meat environment and the meat microflora on survival of the test cultures at 4°C.

The experiment was planned as a multifactorial, repeated measures design to determine the effect of test cultures, broth versus meat as the suspending medium, pH and microbial load of the meat over time of storage (days). The statistical analysis of the data was complicated by the fact that highly significant interaction effects ($P<0.05$) occurred. This seemed to be largely contributed by the fact that different test organisms responded differently to the storage conditions. From the analyses on the data that were partitioned for broth and meat as the suspending media, for each of the test organisms, it was apparent that although

there was often a significant treatment effect, the differences in survival between levels of pH were of no practical significance. The broth studies confirmed that the test organisms survived well in the broths at 4°C, and that changes in pH, equivalent to changes in pH that might be anticipated in meat systems, did not affect the survival of the organisms to any practical extent in the broths.

In meats, the pH was adjusted with ammonium hydroxide, to simulate the growth by-products of the aerobic meat microflora. The increased pH levels of pH 6.0 and 6.5, did not affect the survival of the test cultures any more than the natural meat pH of 5.5 to 5.6. However, there were variable effects attributed to the meats that were subjected to the 24 h vacuum packaged and aerobic treatments before inoculation with the test cultures. These effects appeared to be influenced by the strain of the test organisms rather than by the treatment *per se*. Although highly significant ($P<0.05$) effects were observed with the vacuum packaged and aerobic pretreatments, they had no practical influence on survival of the test organisms.

The conclusions that can be drawn from this study are that the test organisms survived well at refrigeration temperatures (10 and 4°C) when stored in liquid media, but they died at freezing temperatures (-16°C). In contrast, when transferred to a meat environment, they survived better at freezing temperatures (-16 and -40°C) than at refrigeration temperatures.

It has been argued that *E. coli* survives well in raw meats and therefore it is not a good indicator of unsanitary conditions (Goepfert, 1976). The death of *E. coli* at refrigeration temperatures, as seen in this study, suggests that, contrary to the arguments of Goepfert (1976), *E. coli* may still be a valuable indicator organism in raw meats. The low survival of *K. pneumoniae* (*sensu lato*) as compared to *E. coli* has important implications for the concurrent use of *Klebsiella* and *E. coli* as indicators of unsanitary handling and possibly faecal contamination in meats. The greater survival of *K. oxytoca* at freezing temperatures than *E. coli* 11775 or *K. pneumoniae* 13883 indicates that *K. oxytoca* could be more important than *E. coli* when used as indicators to determine the sanitary conditions of frozen meats. The survival of indicator organisms in frozen meats is very important, since it has been reported that food pathogens, for example, *Salmonella*, survive well in these products (Georgala & Hurst, 1963).

The presence of a lactic acid microflora is known to inhibit the growth of enteric microorganisms present in ground meat (Dubois *et al.*, 1979). Vacuum packaging of meats allows the lactic acid bacteria to predominate while suppressing the growth of Gram negative organisms (Foegeding *et al.*, 1983). The object of the pretreatment of vacuum packaging the meat was to observe whether a lactic acid flora would influence survival of the test organisms during the early periods of subsequent aerobic storage. There was

in general, no marked difference between the survival of the *Klebsiella* cultures stored in the "vacuum packaged" meat and those stored in the "aerobic" meat. However, *E. coli* showed considerable loss of viability when stored in "vacuum packaged" meat compared to "aerobic" meat.

After 24 h vacuum packaging, the pH range of 5.6 to 5.7 was still within the normal pH range of fresh meat. It was apparent from these observations, that an aerobic flora was still present in the ground beef after 24 h storage under vacuum packaged conditions, probably because of absorbed oxygen in the meat. Apparently, a storage period of greater than 24 h is necessary for growth of a lactic acid flora. Collins-Thompson *et al.* (1982) reported that a culture of bacteria stored in broth at 15°C under anaerobic conditions increased by almost 3 log cycles within 24 h. This suggests that, ground beef stored in a vacuum package at 10°C, assuming a low initial lactic acid flora (10^0 cells/g), would have contained less than 10^3 cells/g after 24 h as a result of the lower storage temperature. Subsequent aerobic storage of the meat samples at 4°C supported the growth of an aerobic microflora, which may have suppressed any small effect of a beginning lactic acid flora. As a result, the effect of the lactic acid flora on the test cultures may have been lost.

This study indicated that the disappearance of *K. pneumoniae* and *K. oxytoca* in retail meats (Stiles & Ng, 1981) is not primarily the result of phenotypic changes.

Neither is the death of the organisms in meats at low temperatures influenced by pH or the microbial load of the meats. Death of the cells in this study, can be attributed only to the effect of low storage temperatures. Temperature probably damages the cell in some way, interfering with viability. Speculation is that damage is at the site of the cell membrane, disrupting permeability and allowing the leakage of solutes, toxic substances and cell constituents into and out of the cell (Calcott, 1978; Ray & Speck, 1973). Death of *K. pneumoniae* (*sensu lato*) in meats has important implications for the use of these organisms as indicators of unsanitary handling in meats.

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